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14. ABSTRACT

Circulating Mesenchymal Stem Cells (MSCs) originating from the bone marrow have the ability to differentiate into cells of the mesoderm lineage and an innate tropism for tumor tissue in response to the inflammatory microenvironment present in malignant lesions. MSCs have been detected in the perivascular space of many tumors, including those of the prostate, and have been shown to be a critical element in oncogenic progression. MSCs are inherently non-immunogenic, which prevents allogeneic MSCs from being rejected by normal host defense mechanisms. This immune-privileged status, together with their oncotropic properties, makes possible the infusion of allogeneic MSCs into patients for therapeutic purposes, such as the delivery of cytotoxic agents to sites of primary and metastatic prostate cancer. PRX302 is a PSA-activated aerolysin-based protoxin that forms membrane pores and leads to necrosis by a proliferation-independent mechanism at low picomolar concentrations. Importantly, PRX302 binds with low nanomolar affinity to GPI-anchor proteins, which are highly expressed on the surface of all mammalian cells. Therefore, MSCs can be genetically manipulated to express the PRX302 transgene endogenously from a 'safe harbor' locus. Based upon this rationale we hypothesize that human bone marrow-derived mesenchymal stem cells (hMSCs) can be used as a cell-based targeting vehicle to selectively deliver therapeutic agents, such as PRX302, to primary and metastatic sites of prostate cancer, and thus spare host toxicity.

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MSC, prodrug, prostate cancer

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Introduction

Circulating Mesenchymal Stem Cells (MSCs) originating from the bone marrow have the ability to differentiate into cells of the mesoderm lineage and an innate tropism for tumor tissue in response to the inflammatory microenvironment present in malignant lesions. MSCs have been detected in the perivascular space of many tumors, including those of the prostate, and have been shown to be a critical element in oncogenic progression. MSCs are inherently non-immunogenic, which prevents allogeneic MSCs from being rejected by normal host defense mechanisms. This immune-privileged status, together with their oncotropic properties, makes possible the infusion of allogeneic MSCs into patients for therapeutic purposes, such as the delivery of cytotoxic agents to sites of primary and metastatic prostate cancer. PRX302 is a PSA-activated aerolysin-based protoxin that forms membrane pores and leads to necrosis by a proliferation-independent mechanism at low nanomolar concentrations. Importantly, PRX302 binds with low nanomolar affinity to GPI-anchor proteins, which are highly expressed on the surface of all mammalian cells. Therefore, MSCs can be genetically manipulated to express the PRX302 transgene endogenously from a 'safe harbor' locus. Based upon this rationale we hypothesize that human bone marrow-derived mesenchymal stem cells (hMSCs) can be used as a cell-based targeting vehicle to selectively deliver therapeutic agents, such as PRX302, to primary and metastatic sites of prostate cancer, and thus spare host toxicity.

Body

Specific Aim 1: Optimize the recruitment efficiency of allogeneic human bone marrow-derived mesenchymal stem cells (hBM-MSCs) to human prostate cancers in preclinical animal models to maximize their potential as a therapeutic delivery vehicle.

Initially, we had proposed to quantify the efficiency of hBM-MSC homing to prostate cancer xenografted subcutaneously, orthotopically, and intratibially based on quantification using cells fluorescently-labeled with CM-DiI, i.e., counting fluorescent cells (numerator) per field of DAPI-labeled nuclei (denominator). While we were able to qualitatively demonstrate that systemically-infused hBM-MSCs (1×10^6) are able to traffic to prostate cancer xenografts **(Figure 1)**, we were not able to accurately quantify these results using this system. This was due to our inability to resolve the number of cells per fluorescent signal due to inconsistent labeling, both in terms of the staining intensity per cell and the staining pattern, which made absolute quantification using this method difficult and inaccurate **(Figure 1)**.

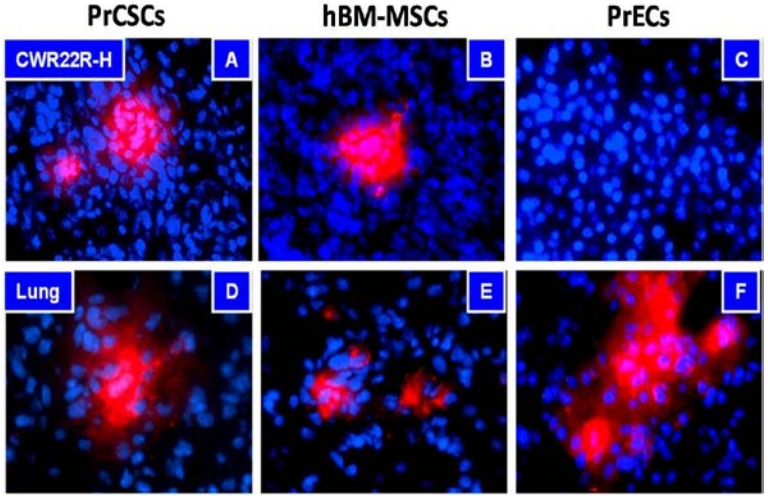

Figure 1: Tumor Trafficking of PrCSCs and hBM-MSCs to Human Cancer Xenografts in Mice. PrCSCs **(A)** and hBM-MSCs **(B)**, but not PrECs **(C)**, traffic to prostate cancer xenografts in vivo following systemic infusion. Fluorescently-labeled (CM-DiI, red) PrCSCs, hBM-MSCs, and PrECs (1×10^6) were infused intravenously (IV) into immunocompromised mice bearing

subcutaneous CWR22RH xenografts (3/group). Four days post-infusion, lungs and tumors were harvested and analyzed by fluorescence microscopy for the presence of CM-DiI-labeled cells. In contrast to the xenografts, all three cell types were found entrapped in the lungs following infusion **(D-F)**. Nuclei counterstained with DAPI (blue). At least three images analyzed per tissue per animal, representative images shown.

To overcome this challenge, we decided to optimize two different technologies for this purpose within our lab. The first of these was designed to allow characterization of the kinetics of MSC trafficking within the murine xenograft models described utilizing a Luc2 expression vector. The Luc2 gene has been engineered to improve mammalian expression and overall sensitivity. The Luc2 gene was amplified from the original pUAS-luc2 plasmid (#24343) obtained from Addgene and cloned into the pLenti-CMV-GFP-Puro expression vector (#658-5) also obtained from Addgene using standard molecular biology techniques to replace the GFP sequence with Luc2. By transducing hBM-MSCs with this lentiviral expression vector we were able to detect a bioluminescence signal from as few as 50 cells in vitro **(Figure 2)**, which is a 4- to 12-fold improvement over the previous generation of luciferase expression constructs (1).

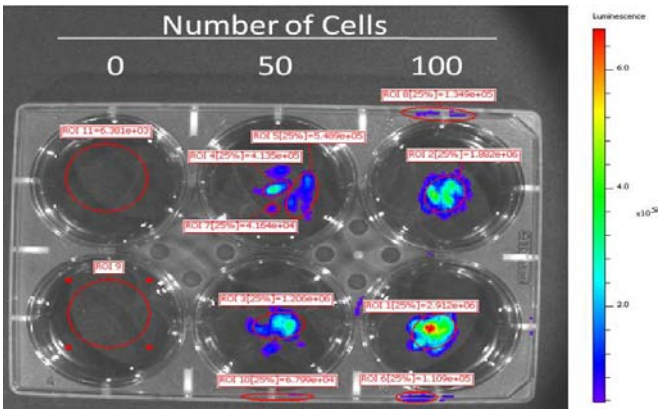

Figure 2: Enhanced sensitivity of bioluminescent signal from hBM-MSCs expressing Luc2. Cells were transduced with a lentiviral expression vector encoding the Luc2 gene. Cells were plated in a six-well plate at the indicated densities in duplicate and allowed to adhere overnight. Luciferin was added, and the bioluminescent signal was quantified using a Xenogen Bioluminescent Imaging System.

An additional challenge that had to be overcome was the sensitivity of MSCs to polybrene (2), a reagent used to enhance the transduction efficiency of viral vectors. The use of polybrene resulted in reduced proliferation and general toxicity that limited the use of the transduced hBM-MSCs for in vivo experiments. To circumvent this problem, polyamine sulfate was substituted for polybrene during the transduction, which has been shown to double transduction efficiencies without the toxicity typically observed with polybrene (3). hBM-MSCs have been successfully transduced with the Luc2 expression vector using an optimized polyamine sulfate transduction protocol for use in the xenograft trafficking experiments. Unfortunately, this system also did not prove sensitive enough to detect infused MSCs in live animals, though there was a small signal detected when the tumors were excised and imaged ex vivo following mechanical digestion.

Quantification of MSC trafficking will be achieved using the second of these optimized techniques, BEAMing, which is a form of digital PCR (4-5). This technology overcomes problems with amplification bias associated with traditional PCR to accurately quantify rare numbers of cells within a complex population at frequencies of less than 1 in 10,000 based upon the presence of mutations or polymorphisms in the DNA sequence. For these purposes, a panel of six SNPs **(Table 1)** from stable genomic regions in prostate cancer has been identified that is suitable for differentiating between donor MSCs and prostate cancer cell lines (6-7). For a panel

of 6 SNPs, this equates to a probability of 1 in 4,049 that the infused MSCs would have an identical profile for all six SNPs analyzed as the xenografted prostate cancer cells.

Gene	Chromosome	Nucleotide Position	SNP ID	Nucleotide change		
				Genotype 1	Genotype 2	Genotype 3
LY9	1	160786670	rs560681	A/A	G/G	A/G
CADM1	11	115207230	rs10488710	C/C	G/G	C/G
N/A	19	39559807	rs576261	A/A	C/C	A/C
PALLD	4	169663615	rs6811238	G/G	T/T	G/T
GABRA2	4	46329655	rs279844	A/A	T/T	A/T
N/A	7	4310365	rs6955448	C/C	T/T	C/T

Table 1: Panel of 6 SNPs capable of differentiating between donor MSCs and prostate cancer cells.

The SNP profile for this panel has already been determined for LNCaP, CWR22Rv1, and VCaP prostate cancer cell lines, in addition to three primary hBM-MSC lines currently in use within the lab **(Table 2)**. These experiments were performed in collaboration with Sysmex-Inostics, Inc. (Hamburg, Germany).

Cells	rs10488710	rs6955448	rs279844	rs576261	rs560681	rs6811238
•LNCaP	C/C	C/T	A/T	A/C	A/G	G/T
•LAPC-4	G/G	C/T	A/A	A/A	A/A	T/T
•VCAP	C/G	C/T	A/A	A/C	A/G	G/G
•MSC-1	C/G	C/T	A/T	A/C	A/G	G/T
•MSC-2	C/C	C/T	A/T	A/C	A/A	T/T
•MSC-3	C/C	C/T	A/A	A/C	A/G	G/T

Table 2: Differentiation of donor vs. host cells using SNPs.

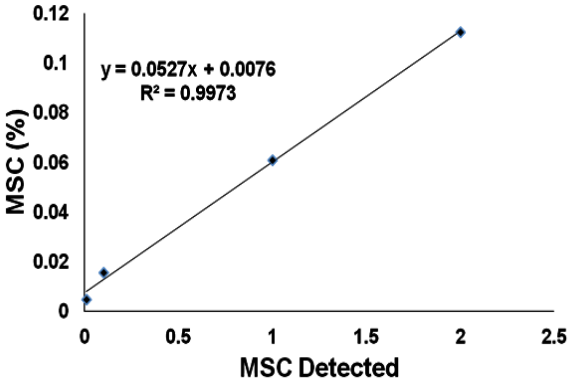

Figure 3: Standard curve of MSCs spiked into prostate cancer cells using BEAMing

Additionally, this panel has been used to generate a "MSC standard curve" for determination of the assay-specific limit of detection. This "MSC standard curve" consisted of a dilution series of MSCs spiked into a suspension of prostate epithelial cells (LNCaP, 100%-0.001%). The sensitivity of the assay allows us to detect donor cells representing as few as 0.01% of the sample **(Figure 3)**. An expanded standard curve in the 0.01-2% range using multiple prostate cancer epithelial cells (LNCaP, LAPC-4, and VCaP) is currently being processed to confirm reproducibility and sensitivity within this range.

Importantly, this sensitivity is in the predicted range for MSCs homing to the human prostate that we determined using an optimized multi-parameter flow cytometry protocol on rapidly dissociated primary prostatectomy tissue (0.01-1.1%, **Appendix I**)(8). Once this validation is

complete, accurate quantification of MSC homing to prostate cancer xenografts in murine hosts will be performed as planned.

Furthermore, a multi-parameter flow cytometry assay has been optimized that can accurately distinguish between human and mouse MSCs. This can also be used to quantify homing of infused human MSCs to prostate cancer xenografts. The results from these two assays will be used to validate each other and to optimize various strategies to enhance MSC homing to sites of prostate cancer.

Specific Aim 2: Genetically modify hMSCs to endogenously express and secrete PRX302 using zinc-finger nuclease and integration-deficient lentiviral technologies to insert the transgene into a "safe-harbor" locus (HIV co-receptor CCR5) within the genome.

This aim was modified to incorporate a better understanding of the delivery platform and enhance the potential for success. Insertion of the PRX302 protoxin (PSA-activated pro-aerolysin) into the PIG-A locus, the first enzyme in the GPI-anchor biosynthesis pathway (9), would prevent self-sterilization by knocking out GPI-anchor synthesis and preventing the toxin from binding to the secreting cell's surface **(Figure 4)**.

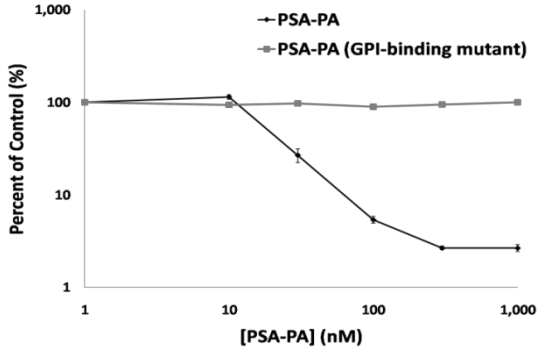

Figure 4: PSA-activated proaerolysin (PRX302) with a mutation in the GPI-anchor binding domain (R624A) has reduced toxicity against LNCaP prostate cancer cells.

Zinc-finger nucleases (ZFNs) targeting the PIG-A locus have previously been generated with the help of our collaborator on this project, Dr. Linzhao Cheng (10). Vectors encoding these PIGA-targeted ZFNs have been obtained through a MTA with Dr. Keith Joung and Harvard Medical School and are currently present within the lab. Additionally, a integration-deficient lentiviral vector (IDLV) encoding the PIG-A homology arms required for homologous recombination-mediated insertion of the PRX302 transgene have been kindly provided by Dr. Linzhao Cheng as well (10). The PRX302 transgene will be subcloned from the pMMB66HE vector into the IDLV vector between the PIG-A homology arms. However, preliminary work in which HEK293T cells were transduced with the PRX302 transgene encoded in a GFP-expressing lentiviral vector **(Figure 5)** demonstrated that the protoxin ran at an aberrant size. This was shown to be the result of post-translational glycosylation due to mammalian expression and was shown to significantly reduce the activity of the toxin. These predicted N-glycosylation sites have been conservatively mutated (N>R) using site-directed mutagenesis, which restored a hemolytic activity **(Figure 6)**. Though hemolytic activity was restored, a significant loss in activity compared to the bacterially-expressed recombinant protoxin was observed. A significant loss in yield was also observed, and therefore, both effects are potentially due to incorrect folding. Current work is underway to determine whether mutation of all five putative glycosylation sites is necessary and whether we can improve both yield and hemolytic activity through more selective mutation of these residues.

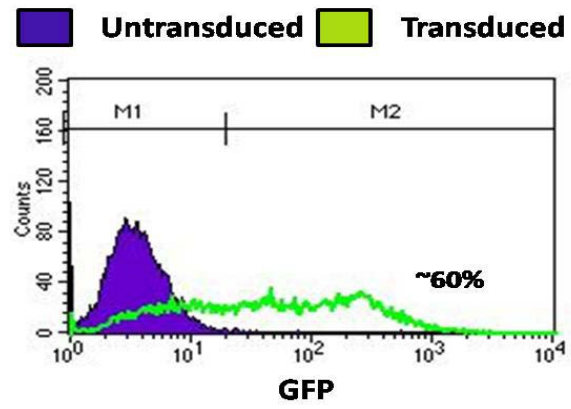

Figure 5: Mammalian cells transduced with a PRX302 expression vector including a GFP reporter. HEK293T cells were transduced with a lentiviral expression vector encoding the PRX302 transgene and a GFP expression cassette driven off a single CMV promoter. A T2A sequence separates the PRX302 and GFP sequences, which results in a 2A-mediated separation of the adjacent proteins via a translational skipping mechanism (11). This vector was generously provided by Dr. Cheng-Lai Fu and Dr. Hans Hammers, who generated the vector by cloning the T2A sequence into the pLVX-AcGFP1-N1 expression vector (PT3994-5) from Clontech.

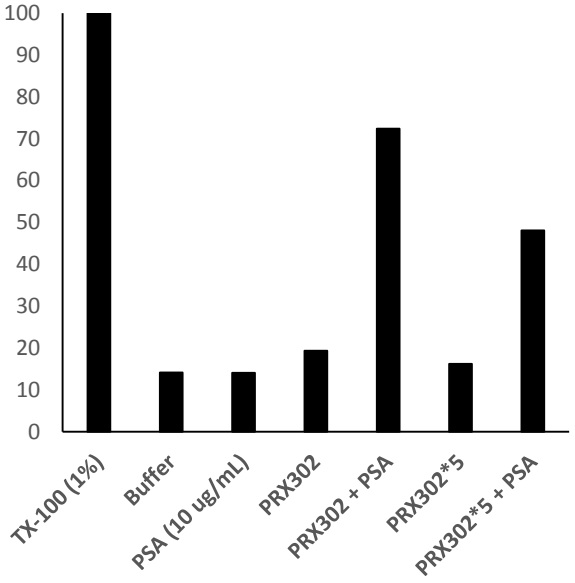

Figure 6: Hemolysis assay demonstrating activity of recombinant PRX302 expressed in mammalian HEK293T cells. Bacterially expressed PRX302 lyses ~70% of RBCs during a 1 hr incubation at 37˚C. In contrast, PRX302 expressed in mammalian cells has no activity (data not shown). Following site-directed mutagenesis to conservatively mutate five putative N-glycosylation sites to arginine (PRX302*5), hemolytic activity was regained by the mammalian expressed protoxin as demonstrated by lysis of ~50% of RBCs during a 24 hr incubation.

Upon successful generation of an optimized mammalian-expressed protoxin, the transgene will be subcloned back into the PIG-A homology IDLV vector. Subsequently, all three vectors (one with the PRX302 transgene flanked by the PIG-A homology arms and vectors for each of the ZFNs targeting the PIG-A locus) will be co-transfected into the target hBM-MSCs. Confirmation of PRX302 insertion into the target locus, and characterization of PRX302 transgene expression and secretion will be analyzed using an optimized western blot protocol and an ELISA assay we recently developed **(Figure 7)**.

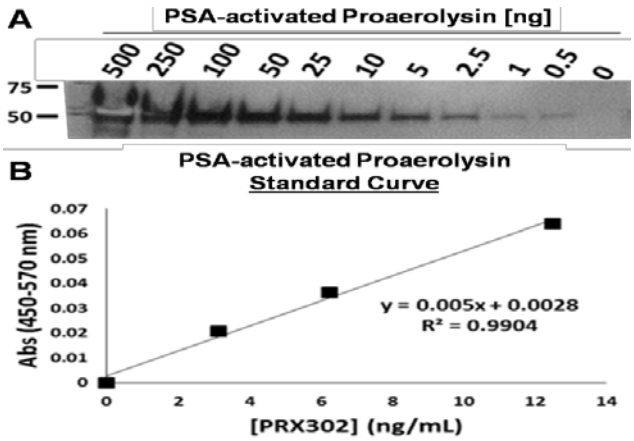

Figure 7: Sensitive detection of PSA-activate proaerolysin by (A) western blot and (B) sandwich ELISA.

These experiments are ongoing and will be completed as initially proposed along with a comparison of the trafficking efficiency of these genetically-modified hBM-MSCs to prostate cancer xenografts relative to their untransfected parental counterparts. Additional experiments will characterize any off-target, non-PIGA integration sites and their associated frequency in this system using both the Surveyor nuclease kit and ultra-deep sequencing.

Specific Aim 3: Evaluate the therapeutic efficacy and host toxicity of genetically modified hMSCs expressing the PRX302 transgene using this optimized homing protocol in preclinical proof-of-principal studies against a series of human prostate cancer xenografts growing in NOG mice.

These experiments have not yet been initiated, but are set to commence over the next several months as results from the ongoing experiments in Specific Aims 1 and 2 are completed.

Key Research Accomplishments

- Tissue digestion and dissociation protocols for primary human prostatectomy tissue have been optimized
- Optimization of flow cytometry-based analyses to characterize co-incident expression of multiple MSC-related markers on a single cell
- Using these optimized protocols, we have demonstrated that MSCs are present in sites of human prostate cancer at a frequency of 0.01-1.1% of the total cells present.
- Optimal growth conditions for the in vitro expansion of MSCs have been delineated
- Stromal cells isolated from primary prostatectomy tissue have multi-lineage differentiation potential (i.e., adipocytes, osteoblasts, and chondrocytes) consistent with an MSC phenotype
- Both hBM-MSCs and MSCs isolated from prostate tissue have tumor-tropic properties following systemic infusion into nude mice bearing prostate cancer xenografts
- Viral transduction protocols have been optimized to both enhance transduction efficiency and reduce toxicity in MSCs
- hBM-MSCs have been transduced with a Luc2 expression vector, and bioluminescent detection has been documented in vitro with as few as 50 cells
- Mammalian cells have been successfully shown to synthesize and secrete the PRX302 protoxin
- The PRX302 protoxin is glycosylated when generated using a mammalian expression system
- Site-directed mutagenesis has been used to conservatively mutate the N-linked glycosylation sites to glutamine residues
- This modified protoxin has been shown to be expressed and secreted by mammalian cells in a functional form that is activated by PSA-dependent manner
- Highly sensitive western blot and ELISA assays for the detection and quantification of PRX302 have been optimized

Reportable Outcomes

Publications:

- **Brennen WN**, Chen S, Denmeade SR, and Isaacs JT. Quantification of Mesenchymal Stem Cells (MSCs) at Sites of Human Prostate Cancer. Oncotarget. 2013 Jan; 4(1): 106-17.
- **Brennen WN**, Denmeade SR, and Isaacs JT. Mesenchymal Stem cells as a vector for the inflammatory prostate microenvironment. Endocr Relat Cancer. 2013 Aug 23; 20(5): R269-90.

Oral Presentations:

- **Brennen WN**. Characterization of Mesenchymal Stem Cells (MSCs) in the Human Prostate. 2012 Dec; Prostate Cancer Young Investigator's Forum, Baltimore, MD.

Poster Presentations:

- **Brennen WN**, Levy O, Ranganath S, Schweizer M, Rosen M, Billet S, Bhowmick N, Denmeade SR, Karm JM, and Isaacs JT. Mesenchymal Stem Cells (MSC) as Cell-based Vectors for PSA-activated Proaerolysin to sites of Prostate Cancer. 2014 Feb; 9th Annueal Johns Hopkins Prostate Research Day, Baltimore, MD.
- **Brennen WN**, Chen S, Denmeade SR, and Isaacs JT. Characterization and Quantification of Mesenchymal Stem Cells (MSCs) in Human Prostate Cancer. 2013 May; SKCCC Research Fellows Day, Baltimore, MD.
- **Brennen WN**, Chen S, Denmeade SR, and Isaacs JT. Characterization and Quantification of Mesenchymal Stem Cells (MSCs) in Human Prostate Cancer. 2013 Mar; Multi-Institutional Prostate Cancer Program Retreat, Ft. Lauderdale, FL.
- **Brennen WN**, Chen S, Denmeade SR, and Isaacs JT. Characterization and Quantification of Mesenchymal Stem Cells (MSCs) in Human Prostate Cancer. 2013 Feb; Prostate Research Day, Baltimore, MD.
- **Brennen WN**, Chen S, Denmeade SR, and Isaacs JT. Detection and Characterization of Mesenchymal Stem Cells (MSCs) in Human Prostate Cancer. 2012 Nov; Society for Basic Urologic Research (SBUR), Miami, FL.
  *2012 Travel Award Recipient
- **Brennen WN**, Denmeade SR, and Isaacs JT. Mesenchymal Stem Cells (MSCs) as a Selective Delivery Vehicle for a PSA-activated Protoxin for Advanced Prostate Cancer. 2012 Mar; Multi-Institutional Prostate Cancer Program Retreat, Ft. Lauderdale, FL.

Additional Funding based on this work:

- PCF Young Investigator Award: "T-cells Engineered to Selectively Deliver a PSA-activated Protoxin to sites of Advanced Prostate Cancer".
- PCF-Movember Challenge Award: "First-in-Man Clinical Studies of Mesenchymal Stem Cell Based Therapy for Prostate Cancer".

- DOD Synergy Award #W81XWH-13-1-0304: “Mesenchymal Stem Cell-Based Therapy for Prostate Cancer”.

Conclusion

Studies arising from this fellowship have led to the quantification and characterization of MSCs present at sites of human prostate cancer, which has recently been published in the open-access journal Oncotarget. Optimal growth conditions for expansion of MSCs in culture have been determined, and the capacity of these cells to traffic to prostate cancer xenografts in vivo has been demonstrated. These experiments are essential prerequisites and have laid the foundation for all future work seeking to develop MSCs as drug delivery vehicles to sites of advanced prostate cancer. Challenges related to the accurate quantification of MSC tumor tropism in vivo and the efficient transduction of this cell type with lentiviral expression vectors have been overcome. Furthermore, proof of PRX302 expression and secretion by mammalian cells in a functional form has been demonstrated following successful mutagenesis of N-linked glycosylation sites – critical components for the success of this therapeutic platform. The therapeutic strategy has been modified from its original incarnation to increase the probability of success by utilizing ZFNs targeting the PIG-A locus, rather than CCR5, and thereby, eliminating the potential for the protoxin-expressing cells to self-sterilize. Furthermore, all of the necessary reagents have been generated or obtained and are currently present within the laboratory to ensure successful completion of the proposed project.

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Mesenchymal stem cells as a vector for the inflammatory prostate microenvironment

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Abstract

Mesenchymal stem cells (MSCs) have an inherent tropism for sites of inflammation, which are frequently present in sites of cancer, including prostatic lesions. MSCs have been defined as CD73/CD90/CD105 triple-positive cells in the absence of hematopoietic lineage markers with the ability to differentiate into multiple mesodermal lineages, including osteoblasts, adipocytes, and chondrocytes. Our group has previously demonstrated that MSCs represent between 0.01 and 1.1% of the total cells present in human prostatectomy tissue. In addition to their multi-lineage differentiation potential, MSCs are immunoprivileged in nature and have a range of immunomodulatory effects on both the innate and adaptive arms of the immune system. MSCs have been detected in an increasing array of tissues, and evidence suggests that they are likely present in perivascular niches throughout the body. These observations suggest that MSCs represent critical mediators of the overall immune response during physiological homeostasis and likely contribute to pathophysiological conditions as well. Chronic inflammation has been suggested as an initiating event and progression factor in prostate carcinogenesis, a process in which the immunosuppressive properties of MSCs may play a role. MSCs have also been shown to influence malignant progression through a variety of other mechanisms, including effects on tumor proliferation, angiogenesis, survival, and metastasis. Additionally, human bone marrow-derived MSCs have been shown to traffic to human prostate cancer xenografts in immunocompromised murine hosts. The trafficking properties and immunoprivileged status of MSCs suggest that they can be exploited as an allogeneic cell-based vector to deliver cytotoxic or diagnostic agents for therapy.

Key Words
- mesenchymal stem cell
- MSC
- inflammation
- prostate cancer
- multipotent stromal cell

Endocrine-Related Cancer (2013) **20**, R269–R290

Introduction

The prostate is the most common organ in the human body to undergo neoplastic transformation when accounting for both benign and malignant lesions. Pathological benign prostatic hyperplasia (BPH) affects >50% of men over the age of 50 years with nonclinical incidence representing a far greater number (Berry *et al.* 1984). Similarly, autopsy studies have demonstrated histological prostate cancer in as many as 50% of men by the age of 50 years with a linear increase in incidence for each subsequent decade of life (Delongchamps *et al.* 2006). The etiology of BPH and prostate cancer is unclear; however, chronic inflammation has been suggested as a

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contributing factor in both (Nelson *et al*. 2003, De Marzo *et al*. 2007, Kramer *et al*. 2007, De Nunzio *et al*. 2011, Sfanos & De Marzo 2012). The prostate, by virtue of its anatomical nature, is among a subset of tissues with a direct route of access to the external environment. Due to this exposure, the prostate routinely comes into contact with potentially infectious agents and frequently contains focal sites of inflammation. Though inflammation is commonly present, external pathogens often cannot be identified within these lesions (De Nunzio *et al*. 2011), suggesting that the inflammatory response persists after the pathogen is cleared or non-pathogenic stimuli are responsible. Other causative agents of prostatic inflammation include dietary and hormonal factors, in addition to chemical and physical irritations resulting from urine reflux and corpora amylacea respectively (De Marzo *et al*. 2007, De Nunzio *et al*. 2011). Furthermore, damage to epithelial cells and glandular structure as a result of these factors can contribute to altered antigen processing and presentation, which can generate an autoimmune response if these peptides are not recognized as 'self' or tolerance is broken (De Nunzio *et al*. 2011, Jackson *et al*. 2012). Independent of the origin, a chronic inflammatory state can arise as made evident by an age-associated persistent presence of an infiltrating leukocyte population (Kramer *et al*. 2007, Nickel *et al*. 2008).

A heuristic model of prostate carcinogenesis suggests that prostate cancer progresses through proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN) precursor lesions prior to malignant transformation (Fig. 1). PIA is defined as focal sites of hyperproliferative epithelial atrophy that are frequently associated with inflammation (De Marzo *et al*. 1999, Nelson *et al*. 2003). PIA is often adjacent to areas of PIN, which are characterized by intraductal cellular proliferation with no evidence of basement membrane and stromal invasion (Clouston & Bolton 2012). During both normal physiological processes and pathophysiological states, dynamic interactions initiated by paracrine mediators occur between the epithelium and cells normally restricted to the stroma, including smooth muscle cells, fibroblasts, bone marrow-derived mesenchymal stem cells (BM-MSCs), and various inflammatory cells. The latter of these have not only been associated with the initiation of prostate cancer but have also been suggested as potential drivers of its progression by virtue of DNA-damaging reactive oxygen species, a variety of immunosuppressive mechanisms, and the secretion of mitogenic and pro-angiogenic cytokines (Nelson *et al*. 2003, De Marzo *et al*. 2007, Sfanos & De Marzo 2012). Once the cancer cells have penetrated the basement membrane, they have direct contact with cells that were previously restricted to paracrine interactions, in addition to direct access to growth factors, survival signals, pro-invasion molecules, and extracellular matrix proteins. In total, these are collectively known as the tumor microenvironment and can have profound effects on cancer progression, malignancy, and therapeutic outcome (Cunha *et al*. 2003, Bissell & Hines 2011, Dayyani *et al*. 2011, Hanahan & Weinberg 2011, Brennen *et al*. 2012, Correia & Bissell 2012).

Chemokines, such as CXCL12 (SDF-1), CCL5 (RANTES), and CCL2 (MCP-1) and the rest of the inflammation-associated secretory milieu, have been shown to recruit MSCs to these sites as a result of the high expression of chemokine and cytokine receptors on their surface (Spaeth *et al*. 2008). Recently, our group has demonstrated that MSCs are not only present at sites of human prostate cancer but also represent 0.01–1.1% of the total cells present in human prostatectomy tissue cores (Brennen *et al*. 2013). MSCs have been shown to be critical mediators of the overall immune response (Caplan 2009, Newman *et al*. 2009, English & Mahon 2011) and, therefore, may contribute to carcinogenesis through a variety of mechanisms, including stimulation of proliferation, angiogenesis, and metastasis, in addition to their immunosuppressive properties (Bergfeld & DeClerck 2010, Klopp *et al*. 2011). These latter properties may be particularly relevant in tumor progression as the cancer cells must escape immune surveillance and clearance to reach their full malignant potential (Fig. 1). Perhaps more importantly, the tumor trafficking properties of MSCs suggest that they could be used to deliver therapeutic or diagnostic agents to sites of prostate cancer, both primary and secondary lesions (Brennen *et al*. 2013).

MSCs: mesenchymal stem cells

Recently, there has been an increasing appreciation for the role of MSCs, also known as multipotent stromal cells, in modulating both innate and adaptive immune responses. These cells were initially characterized by Friedenstein *et al*. (1970) as clonogenic cells in culture that were multipotent stromal precursors. Throughout much of the early literature, these cells were referred to as colony-forming unit fibroblasts or CFU-Fs (Friedenstein *et al*. 1976), until Caplan proposed the term 'Mesenchymal Stem Cells' in 1991 (Caplan 1991). Over the ensuing years, there has been much debate regarding the appropriateness of this terminology (Horwitz *et al*. 2005, Bianco *et al*. 2008,

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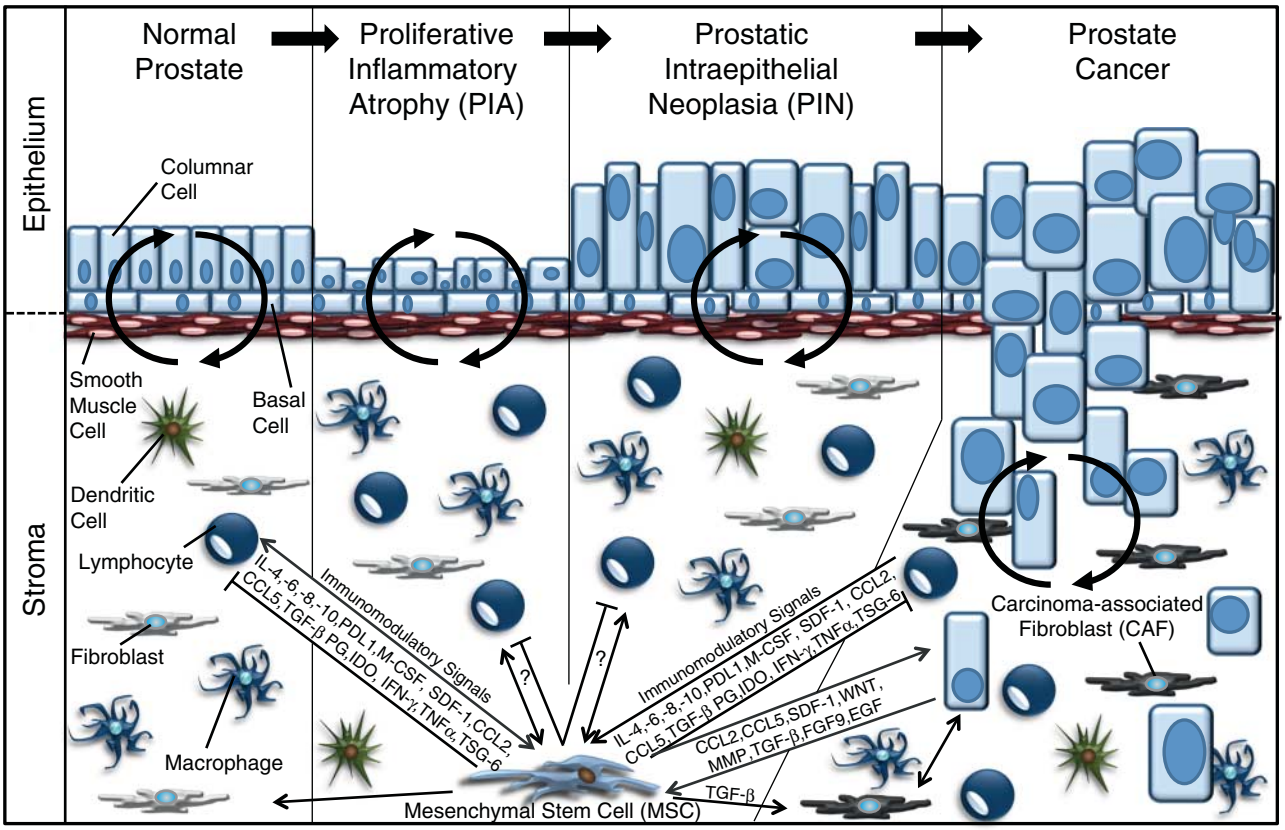

Figure 1

MSCs in the normal and malignant prostate. A heuristic model of prostate carcinogenesis suggests that the normal gland progresses through proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN) stages on its path to malignant transformation. MSCs likely have significant immunomodulatory roles not only in the normal prostate but throughout all stages of prostate cancer tumorigenesis and progression as well. These properties are mediated through the secretion of various chemokines (SDF1, CCL2, and CCL5), cytokines (IL4, IL6, IL8, IL10, M-CSF, IFN-γ, and TNFα) and other bioactive signaling molecules (TGF-β, PG, and IDO) that can indirectly affect carcinogenesis through leukocyte intermediates but also through direct effects on the cancer cells themselves. A full colour version of this figure is available via http://dx.doi.org/10.1530/ERC-13-0151.

Ho *et al*. 2008, Nombela-Arrieta *et al*. 2011); however, this continues to be the accepted consensus in the literature.

The International Society for Cell Therapy has minimally defined MSCs as plastic-adhering multipotent cells of fibroblastoid morphology with the ability to differentiate into cells of the osteogenic, adipogenic, and chondrogenic lineages (Pittenger *et al*. 1999, Dominici *et al*. 2006). MSCs have been further defined based upon the expression of CD90 (Thy-1), CD105 (endoglin), and CD73 (5′-nucleotidase) in the absence of hematopoietic lineage markers, including CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR expression (Dominici *et al*. 2006). Although some are more controversial than others, there is also evidence to suggest that MSCs can differentiate into myocytes (Wakitani *et al*. 1995, Crisan *et al*. 2008), fibroblasts (Lee *et al*. 2010), pericytes (Hirschi & D'Amore 1996, Crisan *et al*. 2008), and neurons (Woodbury *et al*. 2000, Hofstetter *et al*. 2002, Bertani *et al*. 2005, Krampera *et al*. 2007, Phinney & Prockop 2007), although neuronal differentiation appears to be correlated with the age of the donor (Hermann *et al*. 2010, Brohlin *et al*. 2012). The number of MSCs in an individual declines with age, as demonstrated by a 100-fold decrease in the ability of nucleated marrow cells to form colonies (CFU-F), from ~1 in 10^4 in newborns to ~1 in 10^6 in the elderly (Caplan 2009). Furthermore, while MSCs are generally thought to be derived from the mesoderm (Vodyanik *et al*. 2010), there is an initial wave of neuroectodermal MSCs during embryogenesis that arise from the neural crest (Takashima *et al*. 2007, Morikawa *et al*. 2009). Additional evidence suggests that the percentage of MSCs derived from the neural crest declines with age (Takashima *et al*. 2007), which may explain the loss of neuronal differentiation potential observed in MSCs derived from older donors. Additionally, the mesenchymoangioblast was also recently identified as a

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mesoderm-derived precursor able to generate both MSCs and endothelial cells (Vodyanik *et al.* 2010). MSCs have also been isolated from numerous peripheral tissues, including fat, skin, dental pulp, and pancreas (Zuk *et al.* 2002, da Silva Meirelles *et al.* 2006, Zhang *et al.* 2006, Davani *et al.* 2007, Crisan *et al.* 2008, Blasi *et al.* 2011) and are likely present in all tissues at low levels as part of a homeostatic surveillance mechanism.

MSCs: tissue of origin

MSCs isolated from these peripheral tissues are frequently thought of as equivalent to those derived from bone marrow due to significantly overlapping properties; however, there is accumulating evidence to suggest that there are differences between these populations, including their expression profiles (Panepucci *et al.* 2004, Wagner *et al.* 2005, Park *et al.* 2007, Noel *et al.* 2008, Jansen *et al.* 2010, Strioga *et al.* 2012) and differentiation potential (Sakaguchi *et al.* 2005, Musina *et al.* 2006, Strioga *et al.* 2012). These differences may reflect a 'memory', epigenetic or otherwise, associated with distinct signaling events and cellular interactions that occur between MSCs and unique microenvironments. For example, multiple studies have shown that MSCs isolated from fat tissue, or adipose-derived stem cells (ADSCs), have an increased propensity to form adipocytes relative to those derived from bone marrow (Sakaguchi *et al.* 2005, Musina *et al.* 2006). Both synovium- and BM-MSCs seem to have a greater ability to generate chondrocytes than ADSCs (Sakaguchi *et al.* 2005, Afizah *et al.* 2007). Additionally, ADSCs generate osteoblasts with less efficiency relative to their bone marrow-derived counterparts (Sakaguchi *et al.* 2005). However, other studies have shown that both BM-MSCs and ADSCs have equal osteoblast and adipocyte differentiation potential (De Ugarte *et al.* 2003, Krampera *et al.* 2007, Noel *et al.* 2008, Pachon-Pena *et al.* 2011). Our own studies suggest that MSCs from the prostates of young, healthy men selectively lose their adipocyte differentiation ability (W N Brennen, S Chen and J T Isaacs 2013, unpublished observations), while those isolated from cancerous prostates in older men retain their tri-lineage differentiation potential (Brennen *et al.* 2013), which may reflect their more recent exodus from the bone marrow and represent a more naïve commitment status.

Importantly, inter-individual variation in the proliferative capacity and differentiation potential of donor-derived MSCs can make the interpretation of such comparisons difficult, which can be further compounded by differences in optimal culture conditions that are yet to be fully standardized for MSCs obtained from alternative tissue sources (Huang *et al.* 2005, Sakaguchi *et al.* 2005, Wagner *et al.* 2005, Ho *et al.* 2008, Pevsner-Fischer *et al.* 2011, Rada *et al.* 2011, Brennen *et al.* 2013). This variability can be alleviated, in part, by comparing a panel of tissue-specific MSCs isolated from a single individual. Indeed, such studies appear to confirm observations suggesting a restricted differentiation potential related to a tissue-of-origin 'memory' (Sakaguchi *et al.* 2005, Afizah *et al.* 2007). For instance, BM-MSCs have greater chondrogenic potential than ADSCs isolated from the same patient (Huang *et al.* 2005, Afizah *et al.* 2007). Sakaguchi *et al.* (2005) also demonstrated distinct differences in the differentiation efficiencies of patient-matched MSCs isolated from multiple tissues and expanded under similar conditions. Furthermore, gene expression and proteomic analyses of MSCs from different sources have also demonstrated distinct profiles despite significant similarities (Wagner *et al.* 2005, Noel *et al.* 2008, Jansen *et al.* 2010). For example, increased expression of osteogenesis- and angiogenesis-associated genes was measured in BM-MSCs and umbilical cord MSCs relative to each other respectively (Panepucci *et al.* 2004). Minimally, these observations highlight the heterogeneity of isolated MSC populations with regard to their differentiation potential, embryonic lineage, tissue source, and donor age.

MSCs in the clinic

Numerous clinical trials over the last decade were designed to exploit the multipotent differentiation potential of MSCs for a range of pathological conditions, including myocardial infarction (MI), spinal cord injury, and osteogenesis imperfecta. While promising results were obtained in early phase clinical trials, the high hopes for these MSC-based regenerative strategies were largely unrealized in the accompanying phase III trials. Follow-up on these studies suggested a lack of long-term tissue engraftment (<1%) with no evidence of differentiation into the anticipated cell types following systemic administration (Ankrum & Karp 2010). In contrast to the results from *in vitro* differentiation assays, these clinical observations questioned the assumption that MSC's primary role in tissue repair is to reconstitute damaged cell types. However, despite the lack of differentiation, there were positive therapeutic effects observed in select patients from these trials. Concurrent laboratory investigations led to an emerging realization that MSCs function through trophic and immunomodulatory mechanisms based on the secretion of bioactive molecules

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(Krampera *et al.* 2003, Le Blanc *et al.* 2003*b*, Aggarwal & Pittenger 2005, Zimmet & Hare 2005, Iso *et al.* 2007, Prockop 2007, Caplan 2009). MSCs have been shown to secrete numerous growth factors, cytokines, and chemokines, in addition to pro-angiogenic, anti-apoptotic, and anti-inflammatory signals, including transforming growth factor β (TGF-β), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 6 (IL6), regulated on activation, normal T cell expressed and secreted (RANTES), CCL2, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), prostaglandins (PGs), and IL10, to name a few (Newman *et al.* 2009, Zhukareva *et al.* 2010, English & Mahon 2011). Although some groups only detect IL10 in MSC-leukocyte co-cultures (Tse *et al.* 2003, Beyth *et al.* 2005, Rasmusson *et al.* 2005), others have reported the constitutive expression of IL10 by MSCs in monoculture as well (Aggarwal & Pittenger 2005, Barry *et al.* 2005, Coffelt *et al.* 2009, Mougiakakos *et al.* 2011, Technau *et al.* 2011). Additionally, molecular profiling has revealed that MSCs express a large repertoire of cytokine and chemokine receptors that are believed to mediate their trafficking to inflammatory sites (Spaeth *et al.* 2008). The paracrine effects of these secreted molecules likely explain the observed clinical benefits seen thus far and have formed the underlying rationale for the majority of current MSC-based clinical trials designed to treat various inflammatory and autoimmune disorders.

To date, a number of clinical trials have been completed in which *ex vivo* expanded MSCs have been administered for applications as diverse as enhancing cardiac function post-MI, promoting hematopoietic stem cell engraftment, mitigating graft-vs-host-disease (GVHD), and treating a host of autoimmune disorders (Lazarus *et al.* 2005, Le Blanc *et al.* 2008, Hare *et al.* 2009, Garcia-Gomez *et al.* 2010). The most commonly studied application of systemic allogeneic MSCs has been as a means to decrease or prevent GVHD. Two phase III trials enrolling a total of 452 patients have evaluated the efficacy of allogeneic MSCs in patients with GVHD have been completed. While neither trial met its primary endpoint of complete response, there were some clinical benefits observed in those with steroid-refractory GVHD. These results ultimately lead to the approval in Canada of Prochymal, a pre-manufactured, universal donor MSC product, in acute pediatric GVHD (Prasad *et al.* 2011, Osiris Therapeutics 2012). More importantly, as was true with earlier phase studies, no adverse events were noted after infusion of MSCs in any patient being treated on either GVHD protocol. A variety of phase I/II studies testing the effect of MSCs in the setting of MI, chronic obstructive pulmonary disease, liver cirrhosis, lupus, and type II diabetes have also been reported (Hare *et al.* 2009, Jiang *et al.* 2011, Zhang *et al.* 2012, Li *et al.* 2013, Weiss *et al.* 2013). Thus far, no significant MSC-related adverse events have been reported across these various phase I/II studies (Lalu *et al.* 2012). Currently, a number of additional randomized trials are underway utilizing MSCs in a range of different diseases (Garcia-Gomez *et al.* 2010). In summary, the combined results from a large number of trials indicate that i.v. administration of unmodified human BM-MSCs, whether autologous or allogeneic, can be safely administered to patients without producing significant side effects.

MSCs: firemen of the immune system

MSCs are generally thought to be non-immunogenic due to their lack of both MHC-II expression and the associated co-stimulatory molecules (Tse *et al.* 2003). Importantly, MSCs do express low levels of MHC-I, which prevents them from being recognized and lysed by NK cells (Newman *et al.* 2009). Furthermore, the constitutive expression of factor H makes MSCs resistant to complement-mediated lysis (Tu *et al.* 2010). Secretion of factor H extends this protection to other cells in the local microenvironment and represents one of many mechanisms through which MSCs suppress both the innate and adaptive immune responses (Fig. 2). MSCs inhibit the proliferation and activation of NK cells through expression of PGs and indoleamine dioxygenase (IDO; English & Mahon 2011). MSCs secreted PGs have also been shown to suppress mast cell degranulation, trafficking, and tumor necrosis factor α (TNFα) expression (Brown *et al.* 2011), in addition to promoting macrophage M2 polarization (Prockop 2013). Secretion of TSG-6 by MSCs has multiple anti-inflammatory properties, including inhibition of TLR2-induced NF-κB signaling in macrophages by blocking CD44 stimulation, abrogation of neutrophil migration, and suppression of pro-inflammatory protease activity (Lee *et al.* 2009, Prockop & Oh 2012, Prockop 2013). Additionally, MSCs block the secretion of pro-inflammatory cytokines from activated macrophages, prevent the oxidative burst associated with neutrophil function, and suppress eosinophil trafficking to inflammatory tissues (Newman *et al.* 2009, English & Mahon 2011). MSC-derived IL6 inhibits dendritic cell (DC) maturation from monocytes, in addition to suppressing the expression of MHC-II and the CD40 and CD86 co-stimulatory molecules required for T-cell activation (Djouad *et al.* 2007). By blocking DC maturation and antigen presentation, MSCs induce a tolerogenic phenotype in which DCs downregulate the expression of

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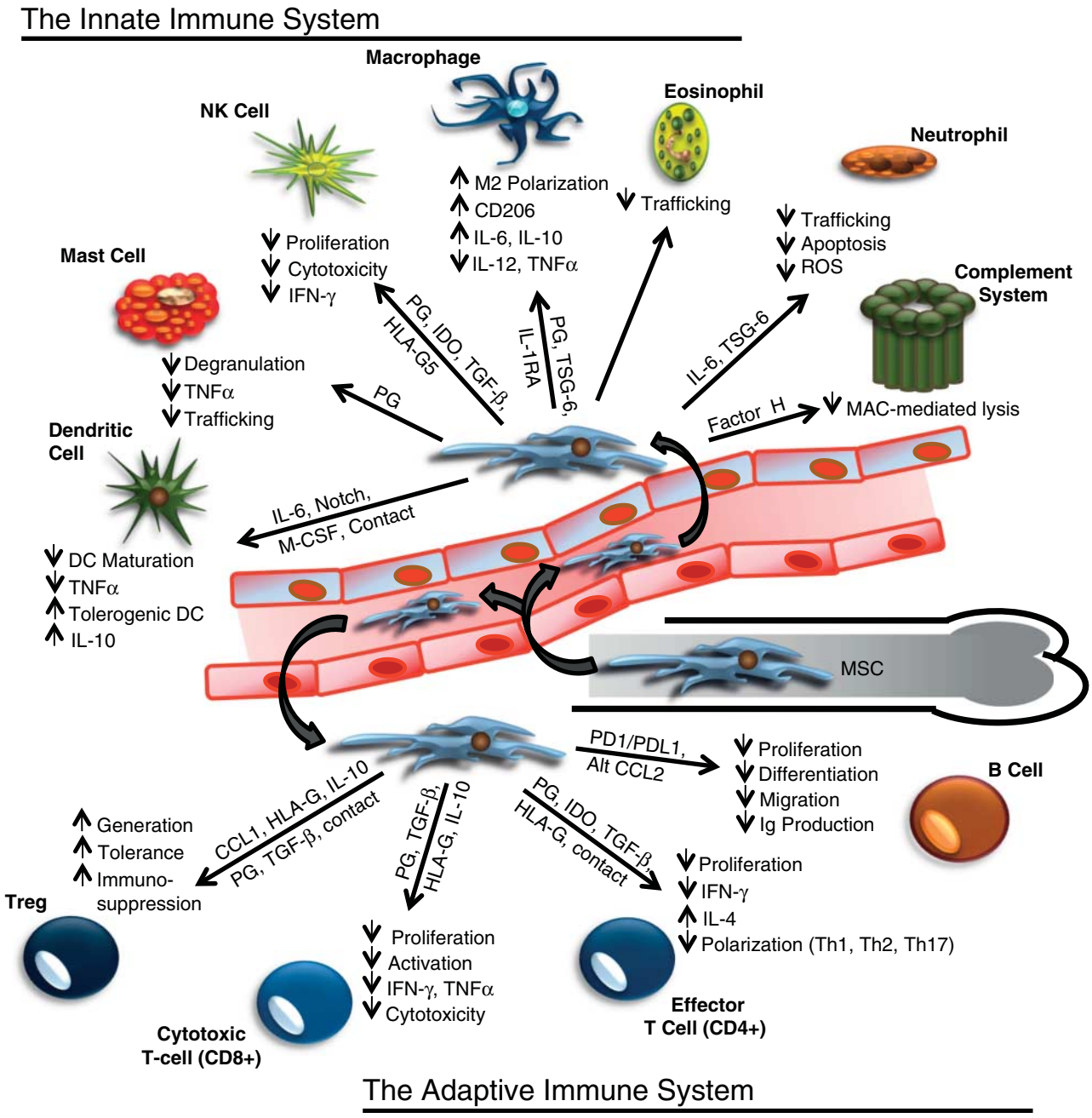

Figure 2

Immunosuppressive properties of MSCs on both the innate and adaptive arms of the immune system. MSC trafficking from the bone marrow in response to an inflammatory stimulus, in addition to MSCs, already present in the local microenvironment can profoundly affect the overall immune response. The immunosuppressive effects of MSCs are mediated through both direct cell contact in some cases, and the secretion of numerous paracrine signals that effect proliferation, survival, trafficking, maturation, polarization, activation, cytotoxicity, and the secretion of additional inflammatory mediators. These effects occur between MSCs and nearly all components of both the innate and adaptive immune system, which suggests that MSCs may represent a central hub in the regulatory networks of the immune system. A full colour version of this figure is available via http://dx.doi.org/10.1530/ERC-13-0151.

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pro-inflammatory cytokines, such as TNFα, while upregulating the expression of IL10 and other anti-inflammatory cytokines (Aggarwal & Pittenger 2005).

Inhibition of MHC-II-mediated antigen presentation by DCs prevents T-cell activation and proliferation. Furthermore, MSCs promote the generation of regulatory T cells; suppress Th1, Th2, and Th17 polarization; and inhibit the proliferation and activation of cytotoxic T cells (CTLs); thereby, shifting the T-cell response to an immunosuppressive state (Newman *et al*. 2009, English & Mahon 2011). Multiple studies have also demonstrated that MSCs inhibit B-cell activation, proliferation, migration, and immunoglobulin expression (Corcione *et al*. 2006, Newman *et al*. 2009). These effects are mediated by both soluble factors and direct cell–cell contact; the latter of which activates programmed death pathway-1 (PD-1) signaling and is at least partially responsible for the attenuation of B-cell proliferation and altered cytokine receptor expression in mice (Augello *et al*. 2005). These observations suggest that a primary physiological function of MSCs is to promote an immunosuppressive microenvironment (Fig. 2). This MSC-mediated immunosuppression likely represents a critical negative feedback mechanism to prevent unchecked chronic inflammation. Together with other regulatory mechanisms, this negative feedback helps to prevent an uncontrolled self-reinforcing 'cytokine storm', or hypercytokinemia, that can lead to increased vascular permeability, tissue edema, autoimmune disorders, fibrosis, acute respiratory distress syndrome, organ failure, cancer, or even death in extreme cases (Osterholm 2005, La Gruta *et al*. 2007).

MSCs: immunoprivileged or not?

While MSCs are traditionally thought to be non-immunogenic and immunosuppressive due to the properties described above, some recent studies have suggested that MSCs may be immunogenic under certain conditions (Eliopoulos *et al*. 2005, Nauta *et al*. 2006, Huang *et al*. 2010). The rejection of allogeneic MSCs in immunocompetent MHC mismatched mice was associated with an increase in infiltrating CTLs, natural killer T cells, and NK cells (Huang *et al*. 2010). Additionally, while syngeneic murine MSCs were associated with tolerance to both donor and recipient antigens in an allogeneic bone marrow transplant model, the same study also demonstrated that transplantation of MHC-matched MSCs and BM into an allogeneic recipient decreased engraftment efficiency (Nauta *et al*. 2006). In contrast, no effect on BM engraftment was observed when MSCs from a third-party donor were used. Furthermore, while allogeneic MSCs only triggered rejection when they were MHC-matched to the BM donor, both third-party and BM-matched MSCs were able to induce a memory T-cell response, which strongly suggests that allogeneic murine MSCs are immunogenic (Nauta *et al*. 2006). Unlike human MSCs, which only express MHC-II following stimulation with interferon (IFN)-γ, the murine MSCs used in these studies express low levels of MHC-II under non-stimulated conditions (Eliopoulos *et al*. 2005). Unsurprisingly, this basal expression of MHC-II renders murine MSCs immunogenic in an allogeneic setting and likely explains the results observed in these studies.

Like humans and other higher order mammals, rat MSCs, in contrast to their murine cousins, do not express MHC-II antigens under basal conditions (Newman *et al*. 2009); however, induction of MHC-II expression was detected following myogenic, endothelial, and smooth muscle differentiation (Huang *et al*. 2010). Furthermore, these differentiated MSCs were cleared from recipient tissues and donor-specific alloantibodies were detected in the serum of recipient rats at 5 weeks post-injection. Similarly, expression of HLA-DR, a MHC-II antigen, is induced following chondrogenic differentiation of adipose-derived human MSCs (ADSCs) *in vitro* (Technau *et al*. 2011). Interestingly, these chondrocyte-differentiated ADSCs continued to express the immunosuppressive HLA-G antigen and secrete IL10, suggesting that they may retain their immunosuppressive properties post-differentiation. Increased immunogenicity following differentiation would potentially explain the lack of data demonstrating long-term engraftment in patients following allogeneic MSC infusion. Indeed, Niemeyer *et al*. (2008) found no evidence of BM-MSCs that were osteogenically induced *ex vivo* prior to infusion in recipient animals; by contrast, undifferentiated BM-MSCs were detected in all recipients following xenotransplantation. Additionally, both allogeneic and autologous BM-MSCs are susceptible to complement-mediated lysis in the presence of serum following *ex vivo* culturing, despite the expression of factor H and other negative regulators (Li & Lin 2012).

Other studies have demonstrated that neither differentiated nor undifferentiated allogeneic MSCs induce a proliferative response in mixed lymphocyte cultures (Le Blanc *et al*. 2003*a*) or in a rabbit model of osteogenesis (Liu *et al*. 2006). Additionally, early studies failed to detect alloantibodies against MSCs in the serum of patients receiving therapeutic doses of allogeneic MSCs (Le Blanc

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et al. 2004, Sundin *et al*. 2007), suggesting that the immunoprivileged phenotype of MSCs remains dominant even if they do undergo differentiation *in vivo*. However, recent studies have reported the presence of anti-donor antibodies in the serum of a minority of patients. Weak alloimmune reactions were detected in 3.7% of patients in the POSEIDON randomized trial comparing allogeneic to autologous BM-MSC therapy for ischemic cardiomyopathy (Hare *et al*. 2012). In a press release reporting the results from Mesoblast's phase 2 trial evaluating MSCs in patients with cardiovascular disease, anti-donor antibodies were detected in 13% of patients (PRNewswire 2011). Importantly, no adverse clinical effects were associated with the presence of alloantibodies in either of these studies.

Ex vivo culturing conditions, particularly with respect to FBS, have been shown to affect the immunogenicity of MSCs and may explain some of the mixed results observed between laboratories (Sundin *et al*. 2007, Newman *et al*. 2009). Multiple trials evaluating the use of autologous MSCs for a variety of conditions have recently been completed or are in progress, and reports on their engraftment efficiency compared with their allogeneic counterparts will address this possibility in a more definitive manner. Importantly, there have been no adverse clinical events, immunological or otherwise, associated with either systemic or local administration of MSCs in the thousands of patients that have been accrued in these trials (Ankrum & Karp 2010, Lalu *et al*. 2012), which emphasizes the overall safety of MSC-based therapeutic strategies. The spontaneous malignant transformation of human MSCs during prolonged expansion *ex vivo* has also been raised as a potential safety concern regarding their clinical use; however, reports on this phenomenon were later corrected or retracted by admissions of contamination in the MSC cultures with other cancer cell lines (Garcia *et al*. 2010, Torsvik *et al*. 2010, Vogel 2010, Klopp *et al*. 2011). Of note, patients enrolled in MSC-based clinical trials often receive multiple doses of $>10^8$ cells, and no transformation of MSCs in these patients have been reported to date. A recent autopsy study of 18 patients receiving infusions of HLA-mismatched MSCs found no evidence of ectopic tissue formation or malignant tumors derived from donor MSCs (von Bahr *et al*. 2012). Furthermore, in eight patients with tissue samples collected more than 50 days post-infusion, low levels of MSC donor DNA ($<1/1000$) were only detected in the lung and kidney of a single patient each. These data corroborate previous clinical observations, suggesting that MSCs have limited long-term engraftment capabilities, which serves to highlight the overall lack of tumorigenic potential for these cells in an allogeneic therapeutic setting.

MSCs: complexities and immunostimulatory properties

There is accumulating evidence to suggest that the interactions between MSCs and immunological effector cells are more complex than those previously appreciated (Fig. 3). For example, MSCs are known to inhibit the IL2-stimulated proliferation of resting NK cells; however, activated NK cells are not only more resistant to MSC-mediated proliferative suppression but have also been shown to lyse both autologous and allogeneic MSCs in the absence of IFN-γ (Spaggiari *et al*. 2006). This lysis occurs as a result of the expression of NK-activating ligands by MSCs (Spaggiari *et al*. 2006). Binding of these ligands to their cognate receptors on the surface of NK cells triggers their recognition and destruction by NK cells, despite the low levels of MHC-I expression on MSCs. Exposure to IFN-γ in an inflammatory microenvironment significantly upregulates MHC-I expression on MSCs and protects them from NK-mediated lysis (Eliopoulos *et al*. 2005, Spaggiari *et al*. 2006). Additionally, IFN-γ-stimulated MSCs also express MHC-II and can function as antigen-presenting cells (APCs; Chan *et al*. 2006, Stagg *et al*. 2006). Interestingly, these antigen-presenting properties are biphasic and only present during a narrow range of IFN-γ concentrations with high levels leading to a decrease in APC functions (Chan *et al*. 2006). MSCs also possess direct antimicrobial activity mediated through the secretion of cathelicidin hCAP-18/LL-37, a peptide with activity against both Gram-positive and -negative bacteria (Krasnodembskaya *et al*. 2010).

The differential activation of TLR signaling in MSCs has also been shown to be a critical mediator of their immunomodulatory properties (Pevsner-Fischer *et al*. 2007, Liotta *et al*. 2008). TLR-2 stimulation suppresses MSC differentiation, while promoting their proliferation and immunosuppressive phenotype (Pevsner-Fischer *et al*. 2007). By contrast, TLR-3 and -4 signaling inhibits this immunosuppressive activity without affecting their differentiation potential (Liotta *et al*. 2008). Activation of different TLR signaling pathways in response to various pathogen-associated molecular patterns (PAMPs) has also been proposed to explain the ability of MSCs to promote tissue repair and control the inflammatory reaction without negatively impacting the ability of the immune system to fight off invading pathogens (English & Mahon

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2011). These observations suggest a model where MSCs would function in a dichotomous manner depending on the nature of the infectious insult and the extent of the immunological response. MSCs would initially behave as APCs to activate an adaptive response following PAMP recognition and IFN-γ stimulation, and the immunosuppressive effects would take dominance during prolonged inflammation with increasing IFN-γ levels (Fig. 3). In further support of this model, the effect of MSCs on lymphocyte proliferation seems to be dependent on the MSC-to-lymphocyte ratio present. Low ratios of MSCs to lymphocytes, as would be seen in the initial phases of inflammation, stimulated lymphocyte proliferation through soluble paracrine mediators, whereas, higher ratios, which may occur after a prolonged inflammatory response, resulted in inhibition of lymphocyte proliferation (Bocelli-Tyndall *et al.* 2009). Evolutionarily, this negative feedback mechanism would serve to limit the immune response and prevent an unbridled leukocytic infiltrate from initiating a self-reinforcing loop of chronic inflammation, which could potentially lead to associated pathological conditions. This parallels a recent model proposed by English & Mahon (2011) in which they describe MSCs as a sort of inflammatory rheostat or 'licensing switch' to modulate the immune response. Additional support for the role of IFN-γ in regulating the immunomodulatory properties of MSCs comes from observations demonstrating that MSC-mediated suppression of T-cell proliferation is enhanced by IFN-γ secreted by activated NK and T cells (Krampera *et al.* 2006, English *et al.* 2007). Furthermore, in contrast to wild-type MSCs, IFN-γ receptor 1-null MSCs were unable to prevent GVHD in mice, suggesting that inflammation and IFN-γ signaling in particular are required for the immunosuppressive effects of MSCs (Ren *et al.* 2008*b*). Importantly, these immunomodulatory properties are probably not dictated by IFN-γ alone but are the result of a complex interplay between IFN-γ, TNFα, and the entire panoply of inflammatory cytokines, chemokines, and signaling molecules present within the local microenvironment.

MSCs and the inflammatory prostate

A model in which MSCs play a primary role in modulating the immune response implies that these cells are present in, or continuously fluxing through, all tissues. Accumulating evidence supports this model. In addition to the bone marrow, MSCs have been isolated from a growing list of tissues, including adipose tissue, skin, muscle, dental pulp, pancreas, intestine, lung, and peripheral blood (Zuk *et al.* 2002, Kassis *et al.* 2006, Zhang *et al.* 2006, Davani *et al.* 2007, Lama *et al.* 2007, Crisan *et al.* 2008, Lanzoni *et al.* 2009, Blasi *et al.* 2011), with all available evidence suggesting that they reside in perivascular niches within all tissues (da Silva Meirelles *et al.* 2006, Crisan *et al.* 2008). While this is a rare, but detectable, population of cells within these tissues under homeostatic conditions, there is a dramatic influx from the bone marrow in response to an inflammatory insult (Spaeth *et al.* 2008, Newman *et al.* 2009). It is well known that the prostate is bombarded with inflammatory and infectious agents throughout an individual's lifetime, with as many as 80% of men showing evidence of a leukocytic infiltrate in their prostate when biopsied (De Marzo *et al.* 2007, Nickel *et al.* 2008, Sfanos & De Marzo 2012). Additionally, the formation of corpora amylacea, which are aggregates of inflammatory proteins, is thought to begin early in life and increase with age, becoming highly prevalent within the prostates of older men (Sfanos & De Marzo 2012). The presence of prostatic inflammation, or prostatitis, likely extends to all men at some point in their lives as many inflammatory stimuli will be resolved without generating overt clinical symptoms.

Therefore, it is unsurprising that MSCs can also be isolated from the prostates of both young and old men (Brennen *et al.* 2013, S Chen, W N Brennen and J T Isaacs 2013, unpublished observations). Lin *et al.* (2007) isolated cells consistent with an MSC phenotype from BPH tissue, a disease characterized by a hyper-proliferative stroma. Despite their ability to differentiate into the myogenic, adipogenic, and osteogenic lineages, the authors concluded that these cells did not represent MSCs due to their inability to generate neural cells, a property later shown to decrease with age (Hermann *et al.* 2010, Brohlin *et al.* 2012), thereby explaining the lack of this particular differentiation potential in BPH cells isolated from older men. Additionally, MSCs incorporate into the re-growing prostates of castrated mice following testosterone supplementation (Placencio *et al.* 2010). In addition to older men with prostate cancer, our own laboratory has cultivated MSCs (CD90+/FAP+/CD105+/CD73+/HLA-DR−) from the prostate of a 20-year-old healthy organ donor (S Chen, W N Brennen and J T Isaacs 2013, unpublished observations), suggesting that MSCs are present in the prostate throughout an individual's lifetime to varying degrees.

Chronic inflammation is thought to be an initiating event for prostatic carcinogenesis (Nelson *et al.* 2003, De Marzo *et al.* 2007). Numerous factors have been implicated in the initiation of an inflammatory microenvironment within the prostate, including diet, infectious agents, physical trauma induced by corpora amylacea, hormonal

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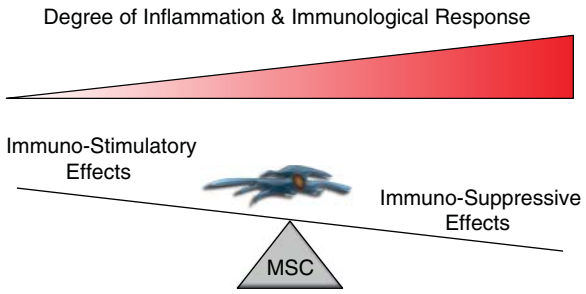

Figure 3
The dichotomous role of MSCs in modulating the immune response depends on the degree of the immunological assault. Evidence suggests that during the initial stages of an inflammatory response, MSCs can behave as antigen-presenting cells and have immunostimulatory effects that activate an adaptive immune response following PAMP recognition and IFN-γ stimulation. As concentrations of IFN-γ, TNFα, and other inflammatory cytokines rise during prolonged inflammation and the lymphocyte-to-MSC ratio increases, the immunosuppressive properties gain dominance and serve as a negative feedback mechanism to prevent unchecked chronic inflammation that can contribute to pathogenesis. A full colour version of this figure is available via http://dx.doi.org/10.1530/ERC-13-0151.

changes, and urine reflux (Sfanos & De Marzo 2012). Independent of the cause, the resulting inflammatory signals act as a chemoattractant for circulating BM-MSCs (Fig. 1) due to the extensive array of chemokine and cytokine receptors expressed on their cell surface (Spaeth *et al.* 2008). CXCL12 (SDF-1), CCL5 (RANTES), and CCL2 (MCP-1), in particular, have been shown to be highly overexpressed in prostate cancer (Sun *et al.* 2003, Vaday *et al.* 2006, Fujita *et al.* 2010), all of which have also been implicated in MSC trafficking to inflammatory sites (Spaeth *et al.* 2008). Multipotent MSCs of mouse origin have been isolated from prostate cancer xenografts using a side population assay (Santamaria-Martinez *et al.* 2009). Additional evidence consistent with the presence of MSCs in human prostate cancer includes the characteristic overexpression of CD90 (True *et al.* 2010), a marker of not only MSCs but also endothelial cells, hematopoietic precursors, neurons, thymocytes, and NK cells. Interestingly, in a series of prostate cancer tissue samples with high CD90 expression, these same authors showed a non-comparable increase in CD45-positive cells, suggesting that the increased CD90 expression was not merely due to excessive leukocyte infiltration (Liu *et al.* 2004). Importantly, not all these extra CD90-positive cells are likely to represent bona fide MSCs as this population also includes MSCs at various stages of differentiation, endothelial cells, hematopoietic progenitors, and carcinoma-associated fibroblasts (CAF). Furthermore, while CD90 expression is significantly elevated in malignant prostatic lesions, rare CD90+ cells can also be detected in normal prostate tissue (Zhao & Peehl 2009, True *et al.* 2010), which is consistent with the presence of a small population of MSCs in all tissues. CD90-positive cells have also been identified in cultures isolated from primary human prostatic stromal cells (Zhao & Peehl 2009). While the authors of this study concluded that these cells did not represent MSCs, it should be noted that CD90hi cells were only compared with CD90lo, rather than CD90-negative cells. Additionally, the differentiation potential of these two CD90-positive populations was not investigated. We would suggest that both these populations likely represent MSCs, albeit potentially ones at different stages of differentiation or lineage commitment. Our own studies clearly indicate the presence of MSCs in multiple primary prostate cancer specimens obtained directly from the operating room prior to expansion in tissue culture (Brennen *et al.* 2013). While CD90 expression has been proposed as a potential cancer biomarker (True *et al.* 2010), the relationship between CD90 expression and PIA or PIN, which are believed to be prostate cancer precursor lesions, has not been studied. Coupled with other characteristic MSC markers, this would help to determine whether MSCs traffic to these inflammatory precursor lesions as an early event in prostate carcinogenesis.

MSCs: effects on tumor progression and metastasis

The role of MSCs in the pathogenesis of cancer is complex and likely related to the balance of competing pro- and anti-tumorigenic forces. Numerous mechanisms have been proposed to play a role in the ability of MSCs to promote tumor growth, including stimulation of proliferation, angiogenesis, and metastasis, in addition to the immunosuppressive properties described earlier. Co-inoculation of MSCs with tumor cells has been shown to increase xenograft growth in models of melanoma and lymphoma, in addition to colon, breast, and lung cancer (Bergfeld & DeClerck 2010, Klopp *et al.* 2011). Tumor growth can be further fueled by promoting an increased tumor vasculature through the secretion of pro-angiogenic factors by MSCs, including VEGF, TGF-β, platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) (Bergfeld & DeClerck 2010, Bianchi *et al.* 2011). MSCs are also frequently found in perivascular niches and can promote vessel stabilization through pericyte-like functions (da Silva Meirelles *et al.* 2006, Crisan *et al.* 2008, Bianchi *et al.* 2011). Additionally, MSCs have been shown to enhance the metastatic potential of breast and colon cancer cells in xenograft models

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(Karnoub *et al*. 2007, Klopp *et al*. 2011), in addition to promoting a pro-tumorigenic environment in the bone marrow (Bergfeld & DeClerck 2010). The immunosuppressive properties of MSCs have also been proposed as a mechanism to enable the tumor to escape host immune surveillance (Bergfeld & DeClerck 2010). Extensive reviews on the relationship between MSCs and cancer have previously been published elsewhere (Bergfeld & DeClerck 2010, Bianchi *et al*. 2011, Klopp *et al*. 2011).

While the pro-tumorigenic role of MSCs is more easily understood, there are also a large number of studies demonstrating anti-tumorigenic effects of MSCs for reasons that are less clear (Klopp *et al*. 2011) but include pro-inflammatory effects and the downregulation of survival signals mediated through the Akt and Wnt pathways (Ohlsson *et al*. 2003, Khakoo *et al*. 2006, Qiao *et al*. 2008). An attempt to reconcile these conflicting observations has recently been discussed by Marini and colleagues, who conclude that there is currently no clear explanation for these divergent findings (Klopp *et al*. 2011). The dichotomous role of MSCs in the immune system likely plays a role in this tumorigenic response; however, both pro- and anti-tumorigenic effects have been observed in both immunocompromised and immunocompetent animals, suggesting that this relationship is more complex than merely a function of their immunomodulatory properties.

MSCs: role in prostate carcinogenesis

Specifically, with regard to MSCs and prostate cancer, several *in vitro* investigations have attempted to understand how the interactions between these two cell types may contribute to carcinogenesis in both the primary and the metastatic tumor microenvironments (Fig. 1). FGF-9 and paracrine factors secreted by bone metastatic PC3 cells stimulate osteoblastic differentiation of human BM-MSCs, whereas conditioned medium from non-metastatic CWR22Rv1 cells did not (Fritz *et al*. 2011). This is particularly interesting in light of the well-known observation that prostate cancer frequently generates osteoblastic lesions when it metastasizes to the bone. Later studies demonstrated that the pro-osteoblastic effect of PC3-conditioned media (PC3-CM) was due to the presence of epidermal growth factor receptor (EGFR) ligands, which also stimulated the proliferation of human BM-MSCs, but suppressed adipocyte and osteoclast differentiation (Borghese *et al*. 2012). Interestingly, significant levels of new bone formation *in vivo* were only observed when MSCs were injected intra-tibially in the presence of PC3 cancer cells but not in their absence (Chanda *et al*. 2009). PC3-CM also stimulated IL6 and CCL5 secretion by MSCs, the latter of which led to increased cell migration; reciprocally, MSCs not only promoted PC3 proliferation and colony formation but also protected them from docetaxel-induced toxicity through paracrine mediators (Borghese *et al*. 2012). Of note, in response to radiation and cytotoxic chemotherapies, stromal cells in the prostate tumor microenvironment were recently shown to secrete paracrine factors, including WNT16B, that promote the survival of adjacent cancer cells and lead to enhanced therapeutic resistance (Sun *et al*. 2012). Ye *et al*. (2012) have shown that media conditioned by human BM-MSCs not only upregulates MMP-2/-9 expression in PC3 cells but also promotes their migration and invasion via TGF-β signaling pathways. Additionally, oncostatin M was recently shown to induce both TGF-β1 and periostin expression in human ADSCs and promote PC3 adhesion (Lee *et al*. 2013).

To date, there have only been a limited number of studies investigating the effect of MSCs on prostate tumor growth *in vivo*, and the majority of those have demonstrated little to no effect (Table 1). It should be mentioned, however, that most of these studies have utilized the PC3 cell line, and therefore, these analyses should be extended into other models before making generalized conclusions. Khakoo *et al*. (2006) demonstrated that human BM-MSCs suppress tumor growth in a model of Kaposi's sarcoma by inhibiting Akt activation in a cell contact-dependent manner; however, when co-cultured with PC3 cells, these same MSCs had no effect on phospho-Akt levels, nor did they alter xenograft growth in immunocompromised animals. No effect on tumor weight or animal survival was observed in mice bearing PC3 tumors who received three weekly i.v. injections of 2×10^6 human BM-MSCs (Wang *et al*. 2012). Rat BM-MSCs transduced with the herpes simplex virus thymidine kinase gene (HSV-TK) had no effect on PC3 xenograft growth in the absence of ganciclovir treatment (Song *et al*. 2011). Additionally, human BM-MSCs had no effect on tumor take or growth rates when co-injected with DU145 cells (Pessina *et al*. 2011). A study by Zhang *et al*. (2011) demonstrated that rat BM-MSCs injected into already established tumors had no effect on PC3 xenograft growth. Additionally, C3H10T1/2 embryonic murine MSCs co-injected with PC3 cells also had no effect on intratibial tumor growth (Fritz *et al*. 2008). By contrast, Chanda *et al*. (2009) showed that adult murine MSCs injected into already established intratibial PC3 tumors suppressed their growth and promoted bone regeneration, although the effect was less pronounced than when the MSCs were co-injected with the tumor cells simultaneously. Using the

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Table 1 Effects of MSCs on prostate cancer growth in preclinical animal models

Effect on tumor growth	Species (MSC)	Tissue source	Mouse strain	Tumor model	Immunogenicity	MSC:cancer cell ratio (no. of MSCs)	Delivery route	Timing (post-inoculation)	MSC injections (#)	Results	Proposed mechanism	Reference
–	Human	Bone marrow	SCID	PC3	Allogeneic	1:6.7 (3×10^5)	MSC: i.v.; tumor: s.c.	d14, 21, 28+ (35 and 42)	Growth: 3; survival: 5	No effect on tumor growth or survival		Wang *et al.* (2012)
–	Human	Bone marrow	Nude	PC3	Allogeneic	1:1.25 (4×10^6)	MSC: i.v.; tumor: s.c.	d0, 3, and 10	3	No effect on tumor growth		Khakoo *et al.* (2006)
–	Human	Bone marrow	NOD/SCID	DU145	Allogeneic	1:5 (0.4×10^6)	MSC: s.c.; tumor: s.c.	d0 (co-inoculation)	1	No effect on tumor take or growth		Pessina *et al.* (2011)
–	Human	Adipose	Nude	PC3	Allogeneic	1:5 (2×10^5)	MSC: s.c.; tumor: s.c.	d0 (co-inoculation)	1	No effect on tumor growth		Zolochevska *et al.* (2012)
–	Human	Adipose	Nude	PC3-M	Allogeneic	1:1 (1×10^6)	MSC: s.c.; tumor: s.c.	d0 (co-inoculation)	1	No effect on tumor growth		Lee *et al.* (2013)
↓/↑	Human	Adipose	Nude	PC3	Allogeneic	s.c.: 2:5 (1.2–1.6$\times10^6$) i.v. 1:1.5 or 1:1 (2–3$\times10^6$)	MSC: s.c. or i.v. tumor: s.c.	s.c.: d0 (co-inoculation) i.v.: d7 and d18	s.c.: 1 i.v.: 1 or 2	s.c.: increased tumor growth and mortality by a couple of days i.v.: suppressed tumor growth	None proposed	Cavarretta *et al.* (2010)
↑	Human	Adipose	Nude	PC3	Allogeneic	1:2 (1×10^6)	MSC: s.c. (left flank); tumor: s.c. (right flank)	d7	1	Increased tumor growth	CXCL12/CXCR4 axis; increased capillary density; FGF2 expression	Lin *et al.* (2010)
↑	Human	Adipose	Nude	MDA-PCa-118b	Allogeneic	1:10 (1×10^5)	MSC: s.c.; tumor: s.c.	d0 (co-inoculation)	1	Increased tumor growth	None proposed	Prantl *et al.* (2010)
↑	Human	Bone marrow	Nude	PC3	Allogeneic	1:5 (1×10^6)	MSC: s.c.; tumor: s.c.	d0 (co-inoculation)	1	Increased tumor growth	TGF-β signaling	Ye *et al.* (2012)
↑	Human	Bone marrow	SCID	PC3	Allogeneic	1:100 (2×10^3)	MSC: s.c.; tumor: s.c.	d0 (co-inoculation)	1	Increased tumor growth	CXCL16/CXCR6 axis	Jung *et al.* (2013)
↓	Mouse	Bone marrow	SCID	PC3	Allogeneic	5:1 (5×10^5)	MSC: I.T.; tumor: I.T.	d1 or d14	1	Suppressed tumor growth effect less pronounced in established tumors; promoted bone regeneration in the presence of cancer cells	New bone formation restricted growth of cancer cells	Chanda *et al.* (2009)
–	Mouse	Bone marrow	SCID	PC3	Allogeneic	1:1 and 3:1 (0.5 and 1.5$\times10^6$)	MSC: I.T.; tumor: I.T.	d0 (co-inoculation)	1	No effect on tumor growth at either ratio		Fritz *et al.* (2008)
–	Mouse	Bone marrow	C57BL/6	TRAMP-C2	Syngeneic	1:2.5 (2×10^5)	MSC: i.v.; tumor: i.v.	d10	1	No effect on lung metastasis		Ren *et al.* (2008*a*,*b*)
–	Mouse	Adipose	C57BL/6	TRAMP-C2-Ras	Syngeneic	1:5 (4×10^5)	MSC: s.c.; tumor: s.c.	d0 (co-inoculation)	1	No effect on tumor growth		Zolochevska *et al.* (2012)
–	Rat	Bone marrow	Nude	PC3	Allogeneic	1:20 (1×10^6)	MSC: i.v.; tumor: s.c.	d3 and 10	2	No effect on tumor growth		Song *et al.* (2011)
–	Rat	Bone marrow	Nude	PC3	Allogeneic	1:1 (2×10^6)	MSC: I.T.; tumor: s.c.	d14, 21, and 28	3	No effect on tumor growth		Zhang *et al.* (2011)

TRAMP-C2 model, Ren *et al*. (2008*a*) demonstrated that murine BM-MSCs had no effect on lung metastasis when injected 10 days post-tumor cell inoculation. Zolochevska *et al*. (2012) showed that human ADSCs had no effect on PC3 xenograft growth in immunocompromised animals nor did murine ADSC significantly stimulate xenograft growth in the immunocompetent TRAMP-C2-*Ras* model. Lee *et al*. (2013) also demonstrated that co-inoculation of human ADSC had no effect on PC3-M xenograft growth in the absence of oncostatin M.

By contrast, Lin *et al*. (2010) showed that implanted ADSCs were recruited to PC3 xenografts on the opposite flank via the CXCL12/CXCR4 axis where they stimulated tumor growth, at least partially through enhanced angiogenesis and FGF2 expression. A study by Cavarretta *et al*. (2010) also suggested that unmodified human ADSCs co-injected subcutaneously with PC3 cells accelerated tumor growth and mortality by a few days; however, systemically administered ADSCs expressing cytosine deaminase (CD) significantly suppressed PC3 tumor growth even in the absence of 5-fluorocytosine (5-FC) treatment. Additionally, Prantl *et al*. (2010) reported increased tumor growth when MDA-PCA-118b cells were co-inoculated with human ADSCs. In contrast to previous reports, Ye *et al*. (2012) observed a significant increase in tumor volumes when PC3 cells were co-injected with human BM-MSCs. Taichman and colleagues recently reported that human BM-MSC stimulated PC3 xenograft growth when co-inoculated at ratios of 1:100 through a CXCR6/CXCL16-dependent mechanism (Jung *et al*. 2013). They further demonstrated that the recruitment of murine MSC to murine RM1 prostate cancer tumors *in vivo* was CXCL16 dependent and the number of MSCs present in the tumor correlated with tumor growth. Furthermore, CXCR6 signaling in BM-MSC induced their conversion to a CXCL12-expressing CAF phenotype, which has been implicated in prostate cancer metastasis (Jung *et al*. 2013).

These conflicting results regarding the influence of MSCs on prostate cancer growth may be due to differences in the ratio of MSCs to tumor cells, the absolute number of MSCs injected, or the timing of their administration relative to tumor inoculation. Furthermore, there does not seem to be a clear relationship between the immunogenicity of the MSCs and tumor cells used nor the immunological status of the xenograft hosts (Table 1). Of note, a recent study by Marini and colleagues suggested that local ADSCs were more likely to be integrated into the fibrovascular network of the early tumor, whereas their bone marrow-derived counterparts were more likely to be localized to the tumor periphery where they may play a role in tissue remodeling and metastasis (Kidd *et al*. 2012). Interestingly, there does seem to be a higher incidence of pro-tumorigenic effects observed in experiments using ADSCs compared with BM-MSCs in the prostate-specific studies described earlier and in those reviewed by Klopp *et al*. (2011), but this is not exclusively true.

Both ADSCs and BM-MSCs have also been shown to give rise to CAF (Fig. 1; Mishra *et al*. 2008, 2009, Paunescu *et al*. 2011, Kidd *et al*. 2012, Jung *et al*. 2013), which have been implicated in nearly all stages of prostate cancer carcinogenesis, including initiation, progression, invasion, and metastasis (Chung 1991, Olumi *et al*. 1999, Bhowmick *et al*. 2004, Franco *et al*. 2010, Giannoni *et al*. 2010, Brennen *et al*. 2012, Li *et al*. 2012). Human prostate-derived CAF co-implanted with initiated but non-tumorigenic human prostate epithelium into immunocompromised murine hosts significantly enhances tumor growth (Olumi *et al*. 1999). Loss of TGF-β responsiveness in fibroblasts through genetic manipulation results in murine PIN-like lesions (Bhowmick *et al*. 2004) and promotes mixed bone lesions in intratibial models of metastasis (Li *et al*. 2012). Conditioned media from activated fibroblasts promotes epithelial-to-mesenchymal transition in PC3 cells *in vitro*, in addition to stimulating invasiveness and prostasphere formation (Giannoni *et al*. 2010). These same authors went on to demonstrate that prostate-derived CAF enhanced PC3 aggressiveness *in vivo* by promoting tumor formation and facilitating lung micrometastases (Giannoni *et al*. 2010). The role of CAF in the progression of tumors from multiple tissues, including breast, colon, and pancreas in addition to the prostate, are well described and have been extensively reviewed elsewhere (Kalluri & Zeisberg 2006, Orimo & Weinberg 2006, Franco *et al*. 2010, Shimoda *et al*. 2010). Our own studies suggest that CAF derived from human prostates are enriched in MSCs (Brennen *et al*. 2013). These seemingly contradictory observations regarding the well-known tumor-promoting properties of CAF and the lack of any effect in the majority of the studies described earlier using MSCs serves to further reinforce the idea that MSCs isolated from different compartments have divergent phenotypes. Perhaps unsurprisingly, this implies that prostate-derived CAF are different than the BM-MSCs from which at least a subset of them is derived. These differences likely arise as a result of their developmental origin and distinct signaling events received through interactions with the tissue and tumor microenvironments in which they are found. Additionally, these CAF may pass through an ADSC intermediate stage depending on their mode of recruitment, which may further add to

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the complexity and heterogeneity observed in the phenotypic and functional differences observed in these cells (Kidd *et al*. 2012).

MSCs: tumor-targeting vectors

Available evidence strongly suggests that the inherent tropism of MSCs for tumor tissue can be exploited to deliver therapeutic and diagnostic agents. Indeed, much preclinical work has already been performed in this area using MSCs derived from a variety of species and tissue sources (Ciavarella *et al*. 2011, Shah 2012). In addition to the tumor-targeting properties of MSCs, their immunoprivileged nature suggests that large quantities of these cells can be harvested from a healthy donor, expanded, and manipulated *ex vivo* prior to infusion into multiple allogeneic patients as an 'off-the-shelf' therapy. This latter point not only makes this therapeutic strategy more practical with regard to time and cost but also alleviates ethical considerations related to re-infusing the cancer patient's own (autologous) cells with regard to their potential to influence tumor malignancy.

A common theme of these strategies is to utilize genetic engineering techniques to generate MSCs that express various molecules with anticancer properties, which are then delivered to the tumor by the MSCs via systemic circulation. Generally, these MSC-delivered anticancer agents fall into one of several categories: immunostimulatory agents, oncolytic viruses, growth factor antagonists, pro-apoptotic factors, anti-angiogenic compounds, or prodrug-converting enzymes. Marini *et al*. pioneered the use of adenoviral transduced MSCs to deliver IFN-β to sites of cancer and have demonstrated efficacy in preclinical models of melanoma, breast, and pancreatic cancer (Studeny *et al*. 2002, 2004, Kidd *et al*. 2010). Delivery of IFN-β by genetically engineered MSCs has also shown efficacy in models of prostate bone and lung metastasis (Ren *et al*. 2008*a*, Chanda *et al*. 2009). Additional immunostimulatory agents, including IL2, IL7, IL12, IL18, IL23, and CX3CL1, have also been engineered into the MSC genome and used to treat a variety of preclinical cancer models, such as glioma, melanoma, Ewing's sarcoma, and renal cell carcinoma (Nakamura *et al*. 2004, Elzaouk *et al*. 2006, Duan *et al*. 2009, Gao *et al*. 2010, Gunnarsson *et al*. 2010).

Multiple groups have also begun developing MSCs as delivery vectors for oncolytic viruses (Nakashima *et al*. 2010). Cell-based delivery of oncolytic viruses cannot only enhance the tumor-targeting potential of these viruses but can also reduce their neutralization by shielding them from pre-existing antiviral antibodies (Mader *et al*. 2009, Huang *et al*. 2013). Dembinski *et al*. (2010) demonstrated that delivery of a conditionally replicating fiber-modified adenoviral vector using MSCs reduced off-target infection and systemic toxicity following i.p. injection in a model of disseminated ovarian cancer. The Pereboeva and Curiel groups have also shown increased efficacy and survival following therapy with conditionally replicating adenovirus-transduced MSCs in ovarian xenograft and breast cancer lung metastasis models (Komarova *et al*. 2006, Stoff-Khalili *et al*. 2007).

The delivery of various prodrug-converting enzymes, including carboxylesterases, CD, and HSV-TK, have also generated provocative results in various preclinical models. Co-inoculation of MSCs expressing HSV-TK with PC3 prostate cancer cells inhibited xenograft growth when treated with ganciclovir, but not in its absence (Song *et al*. 2011). Furthermore, systemically delivered MSCs expressing HSV-TK showed efficacy against orthotopic pancreatic and hepatic xenograft growth and reduced the incidence of pancreatic metastasis (Zischek *et al*. 2009, Niess *et al*. 2011). Altaner and colleagues demonstrated that both co-inoculated and systemically administered ADSCs engineered to express CD significantly reduced tumor burden in animals bearing PC3 prostate cancer xenografts following daily doses of 5-FC (Cavarretta *et al*. 2010). This same group has also shown efficacy against HT-29 colon cancer and A375 melanoma xenograft growth *in vivo* using CD-transduced ADSCs (Kucerova *et al*. 2007, 2008). Additionally, MSCs engineered to express carboxylesterase, which metabolizes CPT-11 into an active topoisomerase I inhibitor (SN-38), have shown efficacy against mouse models of glioma (Yin *et al*. 2011, Choi *et al*. 2012).

Additional strategies seeking to utilize the tumor-targeting properties of MSCs include the delivery of pro-apoptotic factors, such as TRAIL (Grisendi *et al*. 2010, Shah 2012); anti-angiogenic agents, such as thrombospondin-1 (van Eekelen *et al*. 2010) and endostatin (Yin *et al*. 2011); and growth factor antagonists, such as NK4 (Kanehira *et al*. 2007). An interesting approach recently described by Spitzweg *et al*. permits both imaging and therapy to be performed using MSCs transfected with the sodium iodide symporter (NIS), which is normally responsible for concentrating iodide in the thyroid (Knoop *et al*. 2011). NIS expression not only resulted in selective accumulation of iodine in hepatocellular tumors in mice, which made both ^{123}I scintigraphy and ^{124}I PET imaging possible but also abrogated xenograft growth following systemic administration of the radionuclide ^{131}I.

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While these strategies have shown great promise in numerous preclinical models, none have entered into clinical trials yet, although the relatively short time frame since their inception precludes any judgment on their eventual clinical potential. In fact, the world's first unmodified MSC therapy only received approval as recently as 2012 in Canada for the treatment of GVHD (Osiris Therapeutics 2012). However, one attribute of these approaches that may ultimately harm their clinical translation is the failure to take into consideration the trafficking of MSCs to multiple sites throughout the body in addition to the tumor following systemic infusion after the initial entrapment in the lung, including the spleen, kidneys, liver, bone marrow, and other sites of inflammation and remodeling (Gao *et al*. 2001, Devine *et al*. 2003, Allers *et al*. 2004, Detante *et al*. 2009, Choi *et al*. 2011, von Bahr *et al*. 2012). This may increase the off-target/non-tumor effects and systemic toxicity associated with these therapies following infusion. One strategy to circumvent these potential off-target effects is the use of MSCs to deliver prodrugs that are activated in a tumor- or tissue-specific manner. As one example, ongoing studies in our own laboratory in collaboration with multiple other groups are seeking to develop MSCs as vectors to deliver prostate-specific antigen (PSA)-activated prodrugs (Denmeade *et al*. 2003) and protoxins (Williams *et al*. 2007) to sites of metastatic prostate cancer using multiple therapeutic platforms, including nanoparticle-loading strategies and genetic manipulation (Brennen *et al*. 2013). In this therapeutic scenario, prodrugs delivered by MSCs to nontarget tissues will not be activated due to the lack of enzymatically active PSA, which is only present in the prostate and at sites of prostate cancer metastases, thereby reducing systemic toxicity. Additionally, Karp and colleagues have demonstrated that MSC homing and engraftment in inflamed tissue can be increased by decorating their surface with proteins involved in leukocyte extravasation (Sarkar *et al*. 2011). Cell engineering strategies such as this and continued optimization of viral transduction methods for MSCs (Lin *et al*. 2012) will help translate these strategies into the clinic more efficiently.

Summary

In summary, MSCs have emerged as critical regulators of the immune response. The role of these cells in both the innate and adaptive immunity is complex and has yet to be fully elucidated. MSCs have a multitude of immunosuppressive properties through effects on nearly every component of the immune system. Additionally, MSCs also have immunostimulatory effects on many of these same components under specific conditions, particularly during the initial phases of an immunological assault. The balance between these competing forces, which is dictated by IFN-γ and the rest of the inflammatory cytokine milieu, plays a role in numerous pathological maladies, including cancer. MSCs and their progeny have a complex role in tumor biology with both pro- and anti-tumorigenic effects being described. While the immunomodulatory properties of these cells certainly play an important role in this relationship, available evidence suggests that the whole story is far more complex and dependent on numerous interactions with other cells present in the tumor microenvironment, both static residents and dynamic infiltrators. However, despite the incomplete understanding of MSC physiology, current data strongly suggest that these cells have an inherent tropism for tumor tissue based on the inflammatory microenvironment frequently present. These tumor trafficking properties, immunoprivileged nature, and expansion capabilities have the potential for exploitation as a cell-based delivery vector for therapeutic and diagnostic purposes. Cell-based treatment modalities attempting to harness the bodies' own physiology for therapeutic benefit have gained traction over the last few years in a variety of diseases and are sure to represent a growing trend in promising anticancer strategies of the future.

Declaration of interest

S R Denmeade and J T Isaacs have licensed PRX302 to and receive royalties from Sophiris Bio, Corp. Both these relationships for S R Denmeade and J T Isaacs have been disclosed and are under the management of the Johns Hopkins University School of Medicine Conflict of Interest Committee.

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Quantification of Mesenchymal Stem Cells (MSCs) at Sites of Human Prostate Cancer

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ABSTRACT:

Circulating bone marrow-derived Mesenchymal Stem Cells (BM-MSCs) have an innate tropism for tumor tissue in response to the inflammatory microenvironment present in malignant lesions. The prostate is bombarded by numerous infectious & inflammatory insults over a lifetime. Chronic inflammation is associated with CXCL12, CCL5, and CCL2, which are highly overexpressed in prostate cancer. Among other cell types, these chemoattractant stimuli recruit BM-MSCs to the tumor. MSCs are minimally defined as plastic-adhering cells characterized by the expression of CD90, CD73, and CD105 in the absence of hematopoietic markers, which can differentiate into osteoblasts, chondrocytes, and adipocytes. MSCs are immunoprivileged and have been implicated in tumorigenesis through multiple mechanisms, including promoting proliferation, angiogenesis, and metastasis, in addition to the generation of an immunosuppressive microenvironment. We have demonstrated that MSCs represent 0.01-1.1% of the total cells present in core biopsies from primary human prostatectomies. Importantly, these analyses were performed on samples prior to expansion in tissue culture. MSCs in these prostatectomy samples are FAP-, CD90-, CD73-, and CD105-positive, and CD14-, CD20-, CD34-, CD45-, and HLA-DR-negative. Additionally, like BM-MSCs, these prostate cancer-derived stromal cells (PrCSCs) were shown to differentiate into osteoblasts, adipocytes, & chondrocytes. In contrast to primary prostate cancer-derived epithelial cells, fluorescently-labeled PrCSCs & BM-MSCs were both shown to home to CWR22RH prostate cancer xenografts following IV injection. These studies demonstrate that not only are MSCs present in sites of prostate cancer where they may contribute to carcinogenesis, but these cells may also potentially be used to deliver cytotoxic or imaging agents for therapeutic and/or diagnostic purposes.

INTRODUCTION

The prostate is subjected to numerous infectious and inflammatory insults over the course of a man's lifetime, ranging from dietary carcinogens to physical trauma to viral and bacterial pathogens [1]. In fact, greater than 80% of men have evidence of inflammation in their prostate at biopsy [2]. Furthermore, prostatitis likely effects all men at some point during their life, at least acutely [1-2]. While many of these inflammatory lesions will be resolved naturally without intervention, a subset of these will go on to develop clinical symptoms as a result of chronic inflammation. Chronic inflammation has been suggested as an initiating event in prostate carcinogenesis as evidence of a leukocytic infiltrate is frequently present at sites of prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA), prostate cancer precursor lesions [1].

Mesenchymal stem cells (MSCs) are adult stem cells that have recently gained attention as potent modulators of both the innate and adaptive immune responses [3-5]. MSCs have been minimally defined by

the International Society for Cell Therapy (ISCT) as adult stem cells of fibroblastoid morphology that can adhere to tissue culture plastic, express CD73, CD90, and CD105 in the absence of hematopoietic lineage markers, including CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR [4, 6-7]. Additionally, these cells have the ability to differentiate into cells of the mesoderm lineage, including adipocytes, chondrocytes, and osteoblasts [6], but may also include additional cell types such as pericytes [4, 8-9], myocytes [9-10], and neurons [11-13], though the latter is the subject of controversy [14-15]. Due to the lack of HLA-DR expression and the associated co-stimulatory molecules, MSCs are immunoprivileged and thus escape immune surveillance [3-4, 16]. Furthermore, MSCs have been shown to mediate immunosuppression through multiple mechanisms involving nearly every component of the immune system, both the innate and adaptive arms [3-5]. MSCs traffic to sites of inflammation through the action of soluble chemokines and cytokines emanating from these lesions [17-19]. MSCs have been shown to express a great number of the cognate receptors for these chemokines and cytokines, which have been shown to mediate their homing properties [17].

This latter point is particularly relevant, because the prostate has frequently been shown to contain sites of inflammation, and prostate cancer expresses high levels of pro-inflammatory stimuli, including CXCR4, CCL5, and CCL2 [18, 20-22]. In 2007, Lin et al. characterized stromal cells from benign prostatic hyperplasia (BPH) tissue that had multi-lineage differentiation potential consistent with MSCs [23]. However, because these stromal cells lacked the ability to differentiate into neurons, the authors concluded that these cells did not represent MSCs [23]. In 2010 and 2012, however, it was demonstrated that the ability of MSCs to differentiate into neuronal cells is highly dependent on the age of the donor [13, 24]. These studies documented that MSCs derived from older donors (>45) lose the ability to differentiate into neuronal cells [13, 24]. Therefore, since the Lin et al. study utilized BPH tissue from patients older than 45, this differentiation potential would be consistent with MSCs derived from older donors. In the data presented herein, we demonstrate that a population of cells can be isolated from primary prostate cancer specimens prior to expansion in tissue culture that is consistent with an MSC phenotype. These primary prostate cancer stromal cells or PrCSCs are

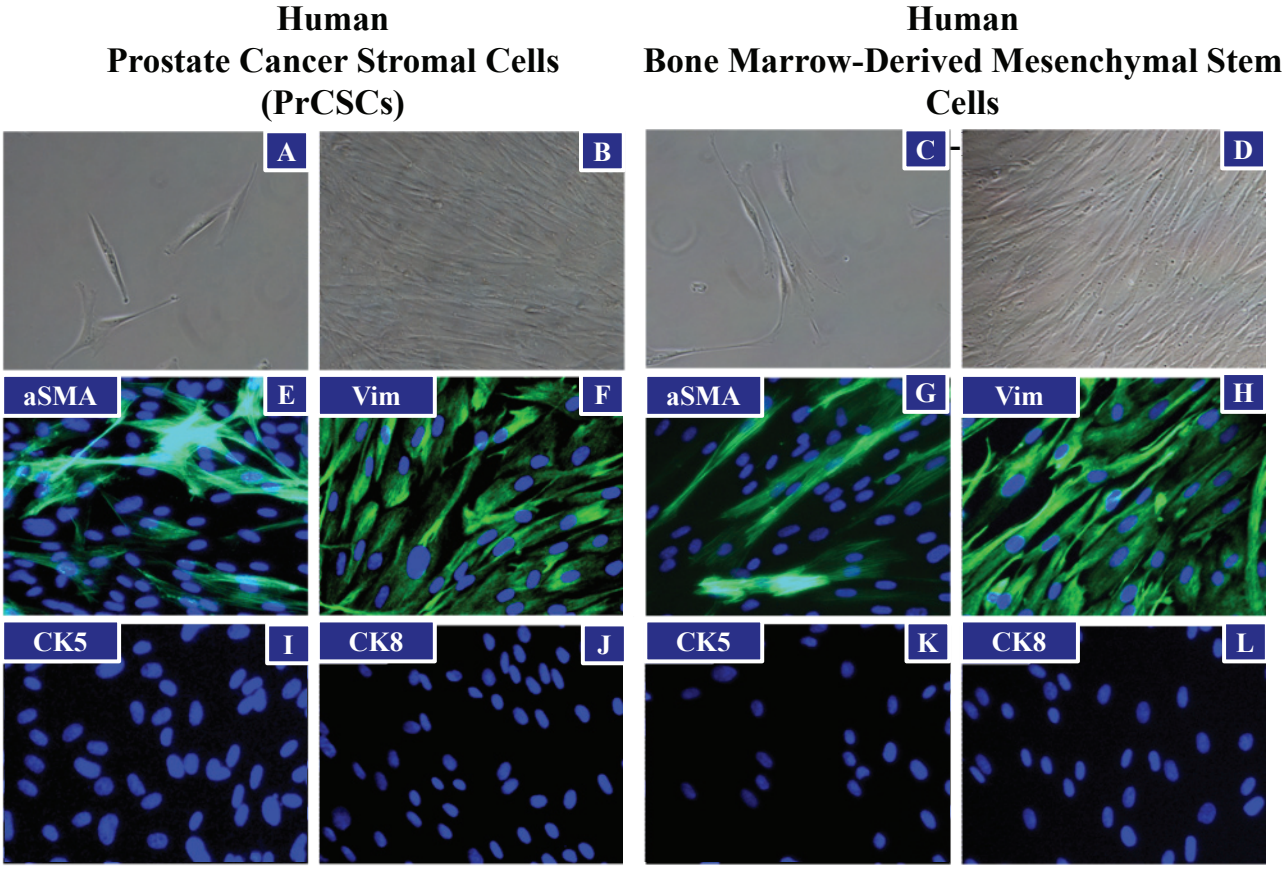

Figure 1: Morphological Similarities between PrCSCs and hBM-MSCs. Prostate cancer-derived stromal cells (PrCSCs) and human bone marrow-derived mesenchymal stem cells (hBM-MSCs) have similar morphologies at low (A and C) and high (B and D) densities (representative phase-contrast images). Both PrCSCs and hBM-MSCs stain positive for mesenchymal markers, alpha-smooth muscle actin (aSMA) (green, E and G) and vimentin (Vim) (green, F and H), but not epithelial markers, cytokeratin 5 (I and K) or cytokeratin 8 (J and L) by immunofluorescence. Nuclei counterstained with DAPI (blue, E-L).

FAP-, CD90-, CD105-, and CD73-positive in the absence of CD14, CD20, CD34, CD45, and HLA-DR expression. Furthermore, a subset of these cells is able to differentiate into osteoblasts, adipocytes, and chondrocytes; thereby, demonstrating their multipotent nature. Like bone marrow-derived MSCs (BM-MSCs), these PrCSCs can traffic to sites of prostate cancer in vivo.

RESULTS

Multi-lineage Differentiation Potential of Human Prostate Cancer-derived Stromal Cells

Tissue cores of human prostatectomy specimens were obtained immediately following surgery, dissociated into a single cell suspension, and placed in tissue culture (RPMI) media supplemented with 10% fetal bovine serum (FBS). From these explanted cells, outgrowth of fibroblast-like prostate cancer-derived stromal cells (PrCSCs) (Figure 1A, B) was observed that had a similar morphology to human bone marrow-derived MSCs (hBM-MSCs) (Figure 1C, D). If a portion of the same cellular suspension was cultured in keratinocyte serum-free media (K-SFM), basal-like prostate-derived epithelial cells (PrECs) were obtained [25-28]. Both hBM-MSCs and PrCSCs stained positive for alpha-smooth muscle actin (aSMA) (Figure 1E, G) and vimentin (Vim) (Figure 1F, H), but not cytokeratins 5 (CK5) (Figure 1I, K) or 8 (CK8) (Figure 1J, L). These results are the absolute opposite of those obtained for PrECs, which are negative for aSMA and Vim, but positive for CK5 and CK8 [25-28]. Similar to hBM-MSCs (Figure 2Q, S, T), differentiation of PrCSCs into adipocytes (Oil Red O-positive) (Figure 2B, G, L), osteoblasts (Alizarin Red-positive) (Figure 2D, I, N), and chondrocytes (Safranin O-positive) (Figure 2E, J, O) was observed if the cells were cultured under the appropriate induction conditions, but not in the uninduced controls (Figure 2A, C, F, H, K, M, P, R). Furthermore, these cells were shown to be fibroblast activation protein $(FAP)^+$, $CD90^+$, $CD105^+$, $CD73^+$, and alpha-smooth muscle actin $(aSMA)^+$ by flow cytometry in the absence of CD45, CD34, CD11b, CD19, and HLA-DR expression (Table 1). In contrast, PrECs do not differentiate into these cell types under the same conditions (data not shown). Importantly,

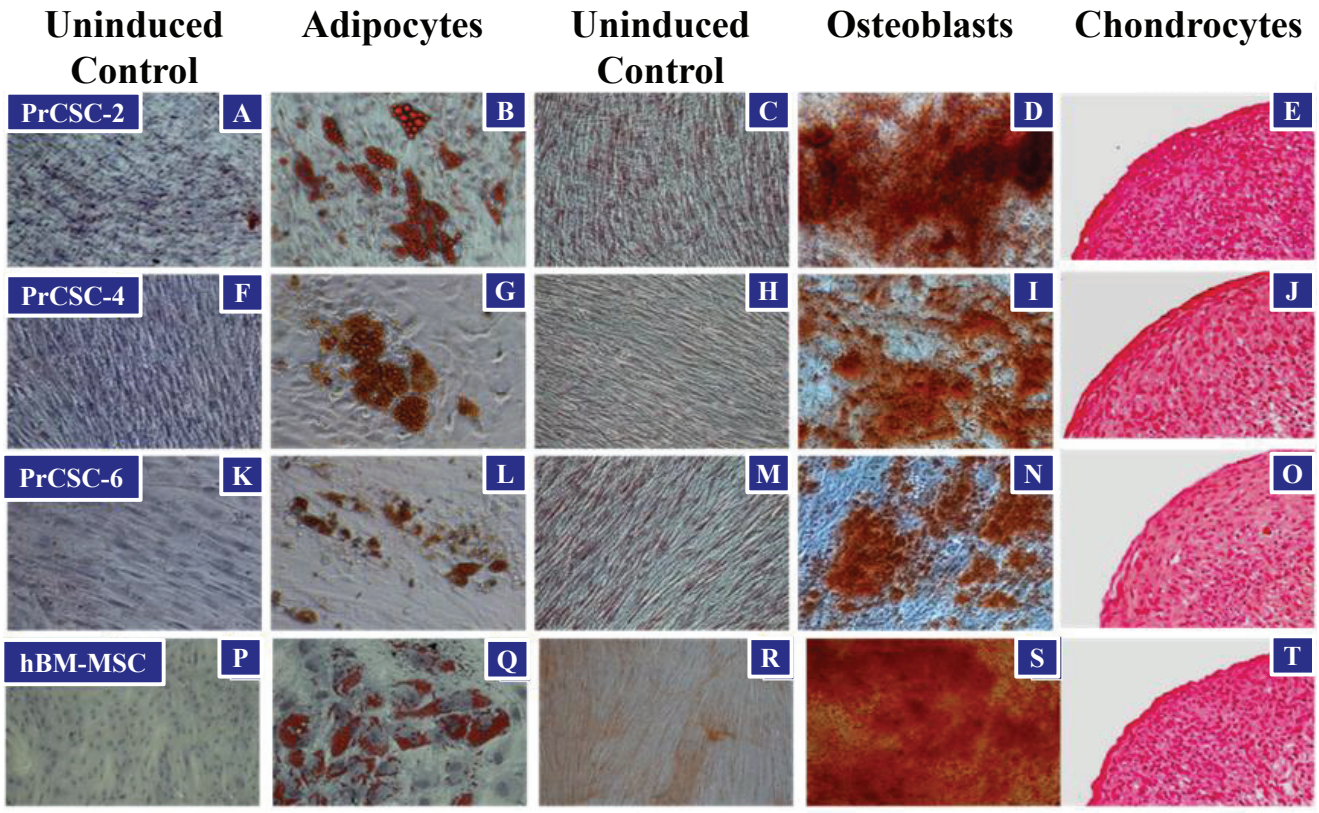

Figure 2: Multi-lineage Differentiation of PrCSCs and hBM-MSCs. PrCSCs derived from multiple patients (PrCSC-2, -4, -6) are able to differentiate into adipocytes (B, G, and L), osteoblasts (D, I, and N), and chondrocytes (E, J, and O) when placed in the appropriate induction media as defined by positive staining for lipid vacuoles (adipocytes, Oil Red O), calcium mineralization (osteoblasts, Alizarin Red S), and glycosaminoglycans (chondrocytes, Safranin-O), respectively. Differentiation indicated by red staining in each. In contrast, no differentiation is observed when these cells are not cultured in the presence of the various inducing factors (adipocytes: A, F, K, and P; osteoblasts: C, H, M, and R). Differentiation into these three lineages is one of the defining characteristics of mesenchymal stem cells as demonstrated by the hBM-MSC positive controls (Q, S, and T).

only a subset of cells within PrCSCs derived from a single donor possesses this tri-lineage differentiation potential (Figure 2A-O). In addition, not all PrCSCs derived from different patients were able to differentiate into all lineages (Table 1). Interestingly, the multi-lineage differentiation potential of the PrCSCs does not appear to correlate with Gleason Score (Table 1).

Quantification of Mesenchymal Stem Cells in Human Prostate Cancer

To eliminate potential artifacts resulting from selection events associated with tissue culture, we optimized a flow cytometry-based assay to directly quantify the number of MSCs present in human prostate cancer samples directly from the patient prior to expansion in culture. Again, tissue cores of prostatectomy specimens were obtained immediately following surgery and digested into a single cell suspension using a combination of mechanical and enzymatic methods. Following labeling with either an MSC phenotyping cocktail (CD73, CD90, CD105, CD14, CD20, CD34, CD45, and HLA-DR) (Figure 3A) or an antibody isotype control cocktail (Figure 3B), these dissociated cells were analyzed by flow cytometry. MSCs within this population of cells were defined as being CD73, CD90, and CD105 triple-positive in the absence of CD14, CD20, CD34, CD45, and HLA-DR labeling (Figure 3). First, cells staining positive for the tested lineage markers were excluded from further analysis. Next, the CD73-positive cells within this lineage-negative population were selected. Finally, the number of MSCs present in the prostatectomy specimens were quantified by determining the number of CD73-positive, lineage-negative cells that were also double-positive for CD90 and CD105 (Figure 3). Of the 10 specimens analyzed in this study, MSCs represented between

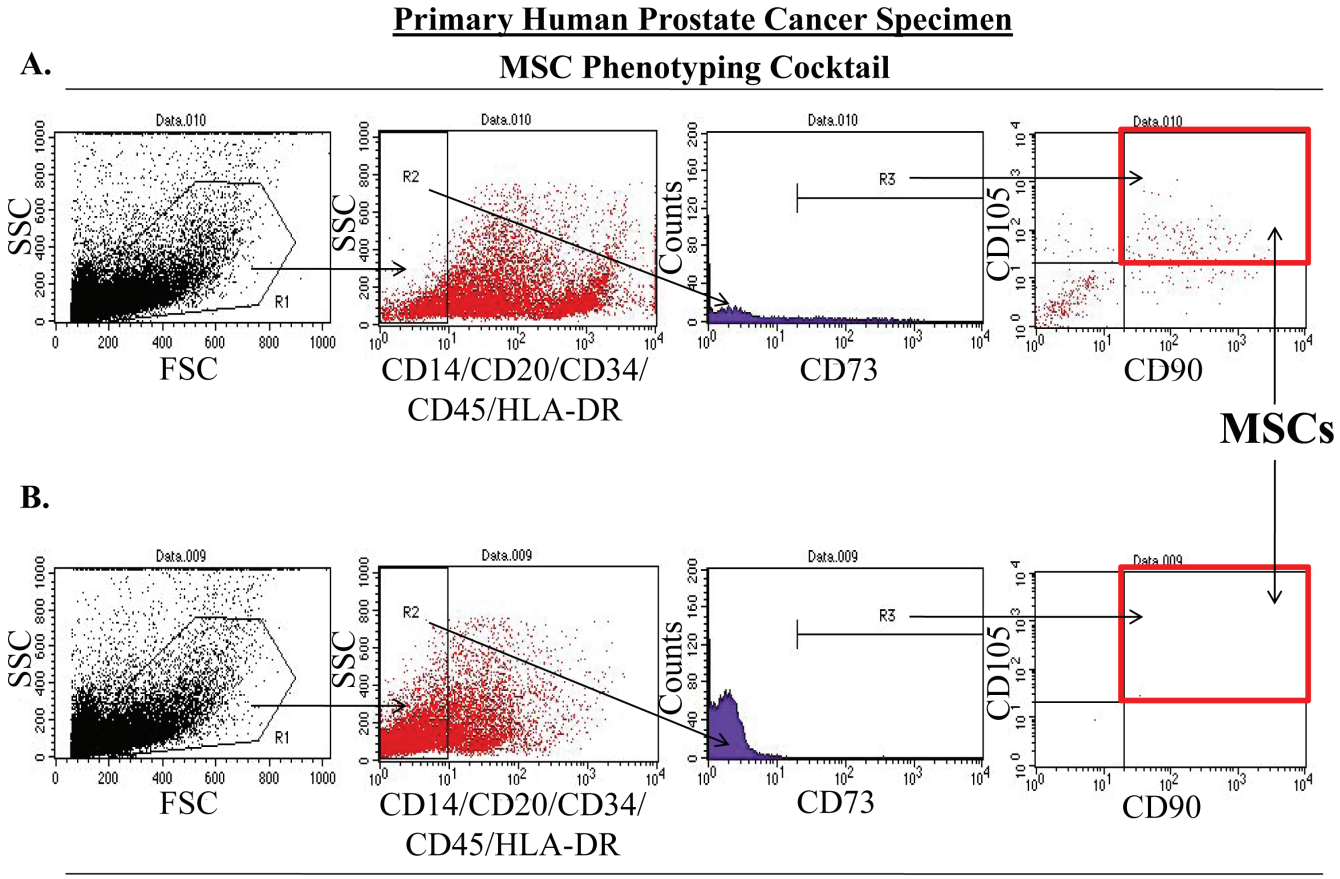

Figure 3: Method for Quantifying MSCs in Primary Human Prostatectomy Samples. MSCs were quantified from primary human prostatectomy specimens using an optimized flow cytometry assay (A-B). Prostatectomy samples were digested into a single cell suspension using a combination of enzymatic and mechanical methods. At least 10,000 cells were initially gated (R1) on the basis forward and side scatter (FSC and SSC, respectively). From this initial population, lineage-negative cells ($CD14^-$, $CD20^-$, $CD34^-$, $CD45^-$, $HLA\text{-}DR^-$) were selected (R2) and analyzed for expression of CD73 (R3). These lineage-negative, CD73-positive cells were further analyzed for the co-expression of CD90 and CD105. MSCs were defined as being lineage-negative and triple-positive for CD73, CD90, and CD105 (red box). Final quantification was performed by subtracting the number of events meeting these criteria in the IgG isotype control cocktail analysis (red box, B) from the events detected in the sample stained with the MSC phenotyping cocktail (red box, A). Importantly, all samples were analyzed within 3 hrs post-surgery.

Table 1: Expression Profile and Differentiation Capacity of PrCSCs and hBM-MSCs.

	FAP	CD90	CD105	CD73	aSMA	CD45	CD34	CD11b	CD19	HLA-DR	Adipo-cytes	Osteo-blasts	Chondro-cytes	Gleason Score
hBM-MSC1	+	+	+	+	+	-	-	-	-	-	+	+	+	N/A
hBM-MSC2	+	+	+	+	+	-	-	-	-	-	+	+	+	N/A
PrCSC-1	+	+	+	+	+	-	-	-	-	-	-	+	-	3+3
PrCSC-2	+	+	+	+	+	-	-	-	-	-	+	+	+	3+3
PrCSC-3	+	+	+	+	+	-	-	-	-	-	+	-	-	4+3
PrCSC-4	+	+	+	+	+	-	-	-	-	-	+	+	+	4+3
PrCSC-5	+	+	+	+	+	-	-	-	-	-	-	-	-	4+3
PrCSC-6	+	+	+	+	+	-	-	-	-	-	+	+	+	4+4

approximately 0.01 and 1.1% of the overall population of cells within the digested prostatectomy tissue (Table 2). As with the multi-lineage differentiation potential of PrCSCs derived from comparable prostatectomy specimens, there does not appear to be a relationship between the quantity of MSCs present in a particular sample and Gleason grade; however, the small number of samples characterized in this analysis preclude any conclusive judgments (Table 2). For comparison, CD31+ endothelial cells represented 1.89% of the cell population in the one prostatectomy specimen analyzed.

Trafficking of Prostate Cancer-derived Stromal Cells & Mesenchymal Stem Cells to Prostate Cancer Xenografts

Additionally, hBM-MSCs are known to home to sites of cancer as a result of the inflammatory microenvironment present within these lesions. To determine whether PrCSCs also retained this ability, fluorescently-labeled cells ($1x10^6$) were administered intravenously (IV) to animals bearing CWR22RH xenografts (3 animals/group). While all cell types tested (PrCSCs, hBM-MSCs, and PrECs) were found entrapped in the lungs at 4 days post-infusion (Figure 4A, B, and C), only the hBM-MSCs and PrCSCs were able to traffic to the prostate cancer xenograft (Figure 4D, E, and F).

DISCUSSION

PrCSCs obtained directly from prostate cancer patients, prior to expansion in tissue culture, express CD90, CD73, and CD105 in the absence of CD14, CD20, CD34, CD45, and HLA-DR as demonstrated using an optimized flow cytometry assay (Figure 3, Table 2). Additionally, at least a subset of PrCSCs retains the ability to differentiate into osteoblasts, adipocytes, and chondrocytes (Figure 2). Therefore, PrCSCs fulfill all of the currently accepted criteria that are used to define MSCs. Importantly, both intra- and inter-patient heterogeneity is apparent in the population of cells isolated according to the current methods. This is demonstrated by the fact that not all PrCSC cultures isolated from different patients retained their multi-lineage differentiation potential (Table 1), and not all cells within a single culture were able to differentiate into all lineages under the appropriate induction conditions (Figure 2). Additionally, each core from each patient was heterogeneous with respect to the amount of cancer present, the number of cancer foci, and the degree of inflammation, all of which likely effect the number of MSCs quantified in any given specimen. Of the 10 prostatectomy specimens analyzed, the number of MSCs ranged from 0.01-1.1% of the overall cell population (Table 2). In comparison, CD31+ endothelial cells, which are known to play absolutely critical roles in tumorigenesis [29-30], represented 1-2% of the cells within sites of prostate cancer. Despite MSCs representing a relatively minor population of cells within the tumors analyzed, their numbers can reach approximately 50% of the endothelial cell content, and therefore, they may potentially play a significant role in prostate tumorigenesis. Interestingly, MSCs are often found in close association with blood vessels where there reside in a perivascular niche [9].

MSCs have previously been shown to influence carcinogenesis in a variety of ways, including promoting proliferation, angiogenesis, and metastasis, in addition to the generation of an immunosuppressive microenvironment [5, 7, 31]. Several studies have also shown MSCs to have anti-tumorigenic properties mediated through immunostimulatory properties and suppression of Akt- and Wnt-mediated survival signals [31-34]. Thus far, only a few studies have examined the role of MSCs in prostate carcinogenesis in vivo, which have predominantly demonstrated no effect on tumor growth [33, 35-41]. Unfortunately, these studies have primarily relied upon the PC3 cell line; therefore, experiments extending these observations into a broader range of models are necessary prior to making any conclusive judgments on MSCs role in prostate carcinogenesis. To further complicate the situation, MSCs have also been shown to give rise to so-

Table 2: Quantification of MSCs in Primary Human Prostatectomy Samples.

Sample	Gleason Score	MSCs (%)
PCa-1	3+3	0.38
PCa-2	3+3	1.10
PCa-3	3+4	0.22
PCa-4	3+4	0.12
PCa-5	3+4	0.01
PCa-6	4+3	1.02
PCa-7	4+3	0.28
PCa-8	4+4	0.14
PCa-9	5+4	0.38
PCa-10	5+5	1.06

called carcinoma-associated fibroblasts or CAFs [42-44], which have been the subject of many investigations into cancer and its relationship with the supporting stroma [45-50]. Our own data suggests that it is relatively easy to expand MSCs from primary human tissue samples under standard culture conditions, and these cells together with their progeny can quickly become a dominant population in the culture. Furthermore, these PrCSCs/MSCs express both aSMA and vimentin (Figure 1), the co-expression of which is commonly used to define reactive fibroblasts or CAFs [50]. This would suggest that many previous studies investigating the role of stromal cells derived from primary human tissue were actually studying MSCs depending on the passage used during the analysis and the frequency of MSCs in the starting population. While the mechanisms underlying the effects of MSCs in carcinogenesis are not fully understood, they are likely related to the complex relationship that exists between MSCs and the immune system [3, 5] coupled with the heterogeneity of tumor microenvironments and the cytokine profile present [18].

Chronic inflammation potentially resulting from a variety of stimuli, including dietary products, infectious agents, corpora amylacea-induced physical trauma, hormonal changes, and urine reflux, is frequently associated with prostate cancer precursor lesions [1-2]. The presence of *Mycoplasma hominis* has also been suggested as a cause of prostate inflammation [51]; however, these results may have been derived from tissue collection artifacts associated with transrectal biopsies [52]. Regardless of the cause, chronic inflammation has been suggested as an initiating event for prostate cancer [1-2]. Additionally, prostate cancers typically express high levels of pro-inflammatory chemokines, including CXCL12 (SDF-1), CCL5 (RANTES), and CCL2 (MCP-1) [20-22]. The expression of these cytokines has been shown to mobilize systemic reservoirs of inflammatory and immunomodulatory cells, including BM-MSCs, which are recruited to prostate cancer lesions [17-18]. MSCs express an extensive array of cytokine receptors, which have been shown to mediate their trafficking to sites of inflammation and cancer [17]. Furthermore, MSCs also secrete a large number of immunomodulatory, growth, and signaling molecules, including TGF-B, GM-CSF, RANTES, CCL2, VEGF, HGF, IL-6, and IL-10 [3-4, 19, 53], which may help to initiate a self-reinforcing loop that may lead to chronic inflammation under pathological conditions and contribute to carcinogenesis. Given the regenerative, immunomodulatory and immunotrafficking properties of MSCs, it is not surprising to find these cells in the prostate during tissue regrowth [54], carcinogenesis (Figure 3, Table 2), and inflammation-associated pathologies, such as BPH [23]. Placencio et al. have previously demonstrated that bone marrow-derived MSCs contributed to prostate regrowth following testosterone supplementation in mice post-castration [54]. Previous work by Lin et al. has also demonstrated that stromal cells consistent with an MSC phenotype from older donors can be isolated from BPH tissue [23]. The authors concluded that these stromal cells did not represent MSCs due to their inability to generate neurons, a property that has been shown to decrease with the age of the donor [13, 24], and therefore, is likely explained by the prevalence of BPH in older men from which the tissue analyzed was obtained. An earlier study comparing CD90hi vs. CD90lo primary stromal cells isolated from prostate cancer patients also concluded that these cells did not represent MSCs [55]. However, it should be noted that the differentiation potential of these cells was not assayed. Furthermore, CD90hi cells

were compared to CD90lo cells rather than CD90neg cells, both of which may represent MSCs at different stages of differentiation and would explain the observed similarities in their expression profiles. The data presented herein clearly demonstrates that there is a rare population of CD90-positive MSCs present in tissue isolated from primary prostate cancer patients (PrCSCs) (Figures 2-3, Table 2).

As described above, there is extensive literature demonstrating that BM-MSCs can home to sites of prostate cancer based upon the inflammatory microenvironment present within these lesions [3, 17-19]. Due to the lack of HLA-DR expression and immunologic co-stimulatory molecules, these cells are non-immunogenic even in an allogeneic setting [3-4, 16]. This suggests that MSCs can be used to systemically deliver therapeutic or imaging agents to both primary and metastatic prostate cancer deposits throughout the body. Additionally, our data suggests that PrCSCs retain this tumor trafficking ability as well (Figure 4), which raises the possibility of using autologous cells derived from a patient's own prostatectomy specimen to target systemic disease; however, ethical concerns related to infusing patients with autologous cancer-associated stromal cells would be of significant concern with this latter approach. Much previous work has attempted to exploit the tumor-trafficking properties of MSCs derived from a variety of non-malignant sources to deliver cytotoxic agents to various solid tumor types with mixed results [56-60]. Importantly, these studies failed to take into account that MSCs traffic to other sites throughout the body, including the lungs, bone marrow, and spleen, in addition to the tumor; therefore, dose-limiting toxicities can be manifested from the delivery of these compounds to peripheral non-target tissues. To circumvent this problem, a prodrug approach exploiting tumor- or tissue-selective activation of a therapeutic compound in which the MSCs were used as a vector to enhance drug accumulation within the tumor would potentially be of greater therapeutic benefit. Additionally, studies by Sarkar et al. have demonstrated that cell engineering approaches can be used to enhance the homing and engraftment efficiency of MSCs in target tissues by mimicking mechanisms of leukocyte extravasation [61].

In summary, primary human prostate cancer harbors a population of cells consistent with MSCs. Stromal cells derived from human prostatectomy specimens (PrCSCs) share an expression profile with MSCs derived from the bone marrow (BM-MSCs) for all cell surface markers analyzed. Like BM-MSCs, these PrCSCs have the ability to differentiate into adipocytes, osteoblasts, and chondrocytes; thereby, demonstrating their multi-lineage differentiation potential. Both BM-MSCs and PrCSCs are able to traffic to prostate cancer xenografts in vivo,

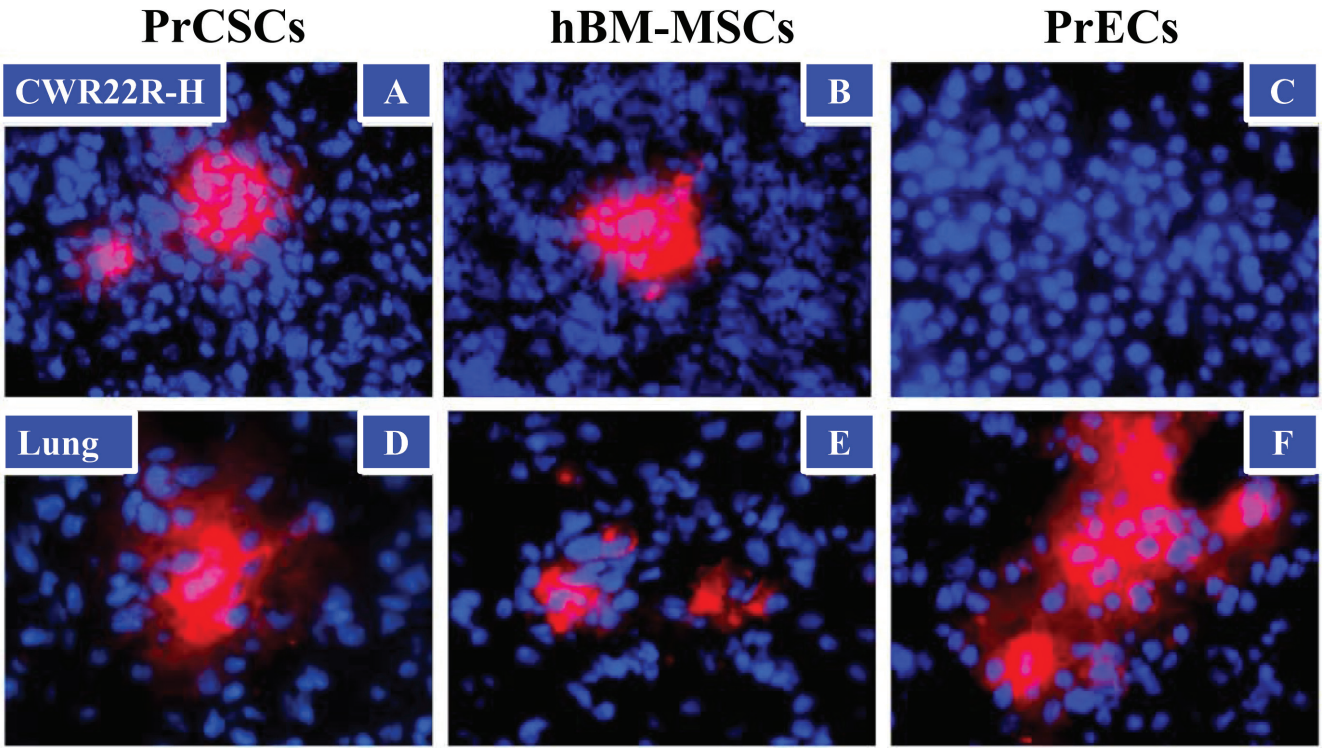

Figure 4: Tumor Trafficking of PrCSCs and hBM-MSCs to Human Cancer Xenografts in Mice. PrCSCs (A) and hBM-MSCs (B), but not PrECs (C), traffic to prostate cancer xenografts in vivo following systemic infusion. Fluorescently-labeled (CM-DiI, red) PrCSCs, hBM-MSCs, and PrECs (1 x 10^6) were infused intravenously (IV) into immunocompromised mice bearing subcutaneous CWR22RH xenografts (3/group). Four days post-infusion, lungs and tumors were harvested and analyzed by fluorescence microscopy for the presence of CM-DiI-labeled cells. In contrast to the xenografts, all three cell types were found entrapped in the lungs following infusion (D-F). Nuclei counterstained with DAPI (blue). At least three images analyzed per tissue per animal, representative images shown.

likely as a result of the pro-inflammatory cytokine and chemokine milieu present. Therefore, MSCs represent a potential drug delivery vector for future therapeutic approaches targeting both local and metastatic prostate cancer.

METHODS

Reagents

Rat anti-human CD11b-APC (clone M1/70.15.11.5), mouse anti-human CD19-PE (clone LT19), mouse anti-human CD34-PE (clone AC136), mouse anti-human CD45-APC (CLONE 5B1), mouse anti-human CD326(EpCAM)-FITC (clone HEA-125), mouse anti-human CD326(EpCAM)-PE (clone HEA-125), mouse anti-human CD326(EpCAM)-APC (clone HEA-125), and mouse anti-human HLA-DR-PerCP (clone AC122) antibodies were purchased from Miltenyi Biotec, Inc. (Bergisch Gladbach, Germany). Mouse anti-human HLA-DR-APC (clone LN3), mouse anti-human CD73-APC (clone AD2), mouse anti-human CD105-PE (clone SN6), mouse anti-human CD326(EpCAM)-biotin (clone 1B7) and mouse anti-human FAP (clone F11-24) were purchased from eBioscience (San Diego, CA). Mouse anti-human CD90-FITC (clone F15-42-1) was purchased from Millipore (Billerica, MA). Mouse anti-human aSMA-FITC (clone 1A4) was purchased from Abcam (Cambridge, MA). Mouse anti-human CK5 (clone XM26) was purchased from Vector Laboratories (Burlingame, CA). Mouse anti-human CK8 (clone LP3K) was purchased from Santa Cruz (Santa Cruz, CA). Mouse anti-human vimentin (clone LN-6) was purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-mouse Alexa Fluor 488, Roswell Park Memorial Institute (RPMI)-1640 medium, keratinocyte-serum free medium (K-SFM), Hank's Balanced Salt Solution (HBSS), L-glutamine, and penicillin-streptomycin were purchased from Life Technologies-Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Gemini Bioproducts (West Sacramento, CA).

Primary Cell Isolation and Tissue Culture

hBM-MSCs were obtained from Lonza (Walkerville, MD). Primary prostate epithelial and stromal cells from patient radical prostatectomy specimens were isolated at our institution in accordance with an Institutional Review Board approved protocol according to previously published protocols [25-28, 62] for the cell cultures used in the differentiation assays, immunofluorescence staining, and cell surface expression studies. hBM-MSCs and PrCSCs were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin in a 5% CO_2, 95% air humidified incubator at 37˚C. PrECs were grown in K-SFM with defined growth factors [25-28] in the same 5% CO_2, 95% air humidified incubator at 37˚C.

Immunofluorescence

Immunofluorescent staining for aSMA, Vim, CK5, and CK8 were performed using the antibodies listed above according to previously published protocols [27-28]. Nuclei are counterstained with DAPI using ProLong Gold anti-fade with DAPI (Invitrogen). Images were captured using a Nikon (Melville, NY) Eclipse Ti Fluorescent scope equipped with a Nikon DS-Qi1Mc camera NIS-Elements AR3.0 imaging software.

Multilineage Differentiation

To assay adipogenic differentiation, 2 x 10^5 cells were plated in a 6-well plate and allowed to reach 100% confluence (3 replicates/cell type) in an incubator with 5% CO_2 at 37˚C. The media was then changed to hMSC adipogenic induction medium (Lonza) supplemented with h-insulin (recombinant), L-glutamine, MCGS, dexamethasone, indomethacin, IBMX (3-isobuty-l-methyl-xanthine), GA-1000. According to the manufacturer's instructions, media was changed every three days alternating between induction and maintenance medium for three complete cycles. After the final cycle, cells remained in the maintenance medium for an additional 7 days prior to evaluation of adipogenic differentiation. Negative control cells were grown in maintenance media only. Adipogenic differentiation was assayed using the lipid stain Oil Red O (Sigma) to identify lipid vacuoles in differentiated cells.

To assay osteogenic differentiation, 3 x 10^4 cells were plated in a 6-well plate and allowed to adhere overnight at 37˚C in an incubator with 5% CO_2 (3 replicates/cell type). According to the manufacturer's instructions, the media was then changed to Osteogenic Induction media (Lonza) supplemented with dexamethasone, L-glutamine, ascorbate, MCGS, b-glycerophosphate. Media was changed every 3-4 days for 21 days. Negative control cells were cultured in RPMI-1640 supplemented with 10% FBS, L-glutamine, penicillin-streptomycin. After 21 days, osteogenic differentiation was assayed by staining for calcium deposits with Alizarin Red S (Sigma).

To assay chondrogenic differentiation, 2.5 x 10^5 cells were centrifuged at 150 x g for 5 min at room temperature and resuspended in 0.5 mL chondrogenic induction medium (Lonza) supplemented with dexamethasone, ascorbate, ITS, GA-1000, sodium pyruvate, proline, L-glutamine, and TGF-B3 in a 15 mL polypropylene conical tube according to the manufacturer's instructions (3 replicates/cell type). The caps were loosened a half-

turn and placed at 37˚C in an incubator with 5% CO_2. The media was changed every 3 days for 21 days while being careful to avoid aspirating the pellet. After 21 days, cell pellets were fixed in formalin and paraffin-embedded for histological processing. Negative controls were cultured in the absence of TGF-B3. Chondrogenic differentiation was assayed by staining for glycosaminoglycans with Safranin-O (Sigma).

Analysis of Cell Surface Markers and MSC Quantification by Flow Cytometry

To analyze cell surface marker expression, prostatectomy cores were dissociated into a single cell suspension as described previously [25-28, 62]. Flow cytometry analyses were also performed as described previously [26-27]. Briefly, all antibody incubations, washes, and flow cytometric analyses were performed in MACS cell sorting buffer (Miltenyi). Antibody labeling was performed at 4˚C for 20 min with a 1:10 dilution of the antibody in a volume of 100 µl per $1x10^6$ cells. The cells were washed in 1 mL cold cell sorting buffer, resuspended in 1.0 mL cell sorting buffer and passed through a 0.2 m filter into a flow analysis tube (BD Biosciences, Franklin Lakes, NJ). Analysis was performed on a BD FACSCalibur flow cytometer.

To obtain cell suspensions for quantification of MSCs by flow cytometry prior to expansion in tissue culture, the following protocol was optimized. Twenty-five 18-gauge biopsy needle cores (C. R. Bard, Inc., Tempe, AZ) were obtained and washed in HBSS. Five randomly selected cores were fixed, paraffin-embedded, and sectioned for H&E staining and pathological confirmation. The remaining cores were digested using a human tumor dissociation kit (Miltenyi) and a gentleMACS dissociator (Miltenyi) according to the manufacturer's instructions. The dissociated cell suspension was then passed through a 70 um pre-separation filter (Miltenyi). The sample was centrifuged at 250 x g for 5 min and resuspended in RBC lysis buffer (Miltenyi) for 10 min at room temperature. The RBC-negative cell suspension was centrifuged at 250 x g for 5 min and resuspended in MACS cell sorting buffer (Miltenyi) to determine cell number and viability by trypan exclusion using a Cellometer Auto T4 (Nexcelcom Bioscience, Lawrence, MA) prior to downstream flow cytometry applications.

All antibody incubations, washes, and flow cytometric analyses were performed in MACS cell sorting buffer (Miltenyi). Antibody labeling was performed at 4˚C for 10 min with a 1:10 dilution of with a MSC Phenotyping Cocktail (anti-CD14-PerCP, anti-CD20-PerCP, anti-CD34- anti-PerCP, anti-CD45-PerCP, anti-CD73-APC, anti-CD90-FITC, and anti-CD105-PE) or Isotype Control Cocktail (Mouse IgG1-FITC, Mouse IgG1-PE, Mouse IgG1-APC, Mouse IgG1-PerCP, and Mouse IgG2a-PerCP) provided in the human MSC phenotyping kit (Miltenyi) in a volume of 100 ul per $1x10^6$ cells according to the manufacturer's instructions. Additionally, anti-HLA-DR-PerCP (Miltenyi) was added to the MSC Phenotyping Cocktail. The cells were washed in 1 mL cold cell sorting buffer, resuspended in 0.5 mL cell sorting buffer and passed through a 0.2 um filter into a flow analysis tube. Analysis was performed on a BD FACSCalibur flow cytometer. All compensation controls were performed using anti-EpCAM antibodies directly conjugated to FITC, PE, APC, or Biotin followed by anti-Biotin-PerCP on aliquots of the same cell suspension to ensure proper gating and instrument settings prior to sample analysis. For sample analysis, cell suspensions labeled with either the Isotype Control or MSC Phenotyping Cocktails were gated (R1) on the basis of forward and side scatter (FSC & SSC, respectively) (Figure 3). Cells gated in R1 were then selected based on being lineage-negative (R2), i.e., negative for CD14, CD20, CD34, CD45, and HLA-DR expression. Next, CD73-positive cells (R3) within these lineage-negative cells were further analyzed for co-expression of CD90 and CD105. MSCs were defined as cells that were triple-positive for CD90, CD73, and CD105 in the absence of the tested lineage markers and quantified by subtracting the number of events, if any, that met these criteria in the isotype control sample. This corrected number was used as the numerator to determine the percentage of MSCs present in the sample. At least 10,000 events were collected in R1, which defined the number of total cellular events and was used as the denominator in the above calculation. Importantly, all samples were processed and analyzed within 3 hrs post-surgery.

Cell Trafficking to Prostate Cancer Xenografts in vivo

Animal studies were performed according to protocols approved by and performed in accordance with the guidelines of the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. For CWR22RH xenografts, 50 mg of minced tumor tissue that had passed through a sterile tissue strainer and washed with HBSS was implanted subcutaneously in 100 ul of 80% Matrigel (BD Biosciences, Sparks, MD) in the flanks of NOG-SCID mice.

To assay tumor trafficking, human PrCSCs, hBM-MSCs (Lonza), or PrECs were fluorescently-labeled ex vivo with CM-DiI (Invitrogen) and washed according to the manufacturer's instructions. Subsequently, $1x10^6$ cells were injected intravenously into NOG-SCID mice bearing subcutaneous CWR22RH tumors (3 mice/group). Animals were euthanized by CO_2 asphyxiation at 4 days post-infusion. The lungs and tumors were harvested from each mouse, flash frozen in VWR Clear Frozen Section

Compound (Radnor, PA), and 4 μm sections were cut on a Shandon Cryotome E (Thermo Scientific, Waltham, MA). Nuclei are counterstained with DAPI using ProLong Gold anti-fade with DAPI (Invitrogen). Images were captured using a Nikon (Melville, NY) Eclipse Ti Fluorescent scope equipped with a Nikon DS-Qi1Mc camera NIS-Elements AR3.0 imaging software.

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Publications:

2014 **Brennen WN**, Drake CG, Isaacs JT. Enhancement of the T-cell Armamentarium as a Cell-based Therapy for Prostate Cancer. Can Res. Under Review.

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Platform Presentations:

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Posters/Abstracts:

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2008 **Brennen WN**, Aggarwal S, Kole T, Rosen DM, Denmeade SR. Fibroblast activation protein (FAP): targeting the reactive stroma as a novel prodrug therapy. Multi-Institutional Prostate Cancer Program Retreat, Newport, RI.

2007 **Brennen WN**, Aggarwal S, Kole T, Yates MS, Rosen DM, Denmeade SR. Fibroblast activation protein (FAP): targeting the reactive stroma as a novel prodrug therapy. General Meeting of the International Proteolysis Society (IPS), Patras, Greece.

2006 **Brennen WN**, Aggarwal S, Kole T, Topaloglu O, Schneider E, Becker R, Denmeade SR. Fibroblast activation protein (FAP): targeting the reactive stroma as a novel prodrug therapy. Prostate Cancer (Special AACR meeting), San Francisco, CA.

2005 Lynch JE, **Brennen WN**, Ottaviani D, Davis GC, Brown ML, Sikes RA. Novel voltage-sensitive sodium channel blockers increase cytotoxicity and decrease metastatic behavior in the LNCaP human prostate cancer progression model in vitro. 96th Annual American Association for Cancer Research (AACR), Anaheim, CA.

2004 Chen Q, Watson JT, **Brennen WN**, Stewart DA, Marengo SR, Decker KS, Kelly RC, Nelson PS, Sikes RA. Molecular profiling of lineage-related androgen-sensitive (LNCaP) and insensitive (C4-2) prostate cancer cell lines. 95th Annual American Association for Cancer Research (AACR), Orlando, FL.

2004 Cooper CR, Sikes RA, Poindexter C, **Brennen WN**, Green J, Capitosti S, Brown ML. A novel compound inhibits the growth of human bone marrow-endothelial cells and bone metastasizing prostate cancer cells. IVth International Conference on Cancer-Induced Bone Diseases, San Antonio, TX.

Teaching Experience:

2005 – present Directly responsible for the immediate supervision and training of eight graduate/medical students during their rotation projects within the lab and three International combined bachelor's/master's degree students from Germany, Russia, and Netherlands respectively, during their traineeships.